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<p>(21) International Application Number: PCT/US99/22668 (22) International Filing Date: 30 September 1999 (30.09.99)  (30) Priority Data:  <table border="0"> <tr> <td>60/102,461</td> <td>30 September 1998 (30.09.98)</td> <td>US</td> </tr> <tr> <td>60/108,109</td> <td>12 November 1998 (12.11.98)</td> <td>US</td> </tr> <tr> <td>60/110,749</td> <td>3 December 1998 (03.12.98)</td> <td>US</td> </tr> <tr> <td>60/113,002</td> <td>18 December 1998 (18.12.98)</td> <td>US</td> </tr> <tr> <td>60/135,426</td> <td>21 May 1999 (21.05.99)</td> <td>US</td> </tr> <tr> <td>60/144,022</td> <td>15 July 1999 (15.07.99)</td> <td>US</td> </tr> </table>  (71) Applicants: LUDWIG INSTITUTE FOR CANCER RE- SEARCH [US/US]; 605 Third Avenue, New York, NY 10158 (US). HELSINKI UNIVERSITY LICENSING LTD. [FI/FI]; OY (FI/FI), P.O. Box 26, FIN-00014 (FI).  (72) Inventors: ERIKSSON, Ulf; Stockholm (SE). AASE, Karin; Stockholm (SE). LEE, Xuri; Stockholm (SE). PONTEN, Annica; Stockholm (SE). UUTELA, Marko; Stockholm (SE). ALITALO, Kari; Helsinki (FI). OESTMAN, Arne; Uppsala (SE). HELDIN, Carl-Henrik; Uppsala (SE). BETSHOLZ, Christer; Uppsala (SE).</p>		60/102,461	30 September 1998 (30.09.98)	US	60/108,109	12 November 1998 (12.11.98)	US	60/110,749	3 December 1998 (03.12.98)	US	60/113,002	18 December 1998 (18.12.98)	US	60/135,426	21 May 1999 (21.05.99)	US	60/144,022	15 July 1999 (15.07.99)	US	<p>(74) Agent: EVANS, Joseph, D.; Evenson, McKeown, Edwards &amp; Lenahan, P.L.L.C., Suite 700, 1200 G Street, N.W., Washington, DC 20005 (US).  (81) Designated States: AE, AU, BA, BB, BG, BR, CA, CN, CU, CZ, EE, GD, HR, HU, ID, IL, IN, IS, JP, KP, KR, LC, LK, LR, LT, LV, MG, MK, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, SL, TR, TT, UA, UZ, VN, YU, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  Published <i>With declaration under Article 17(2)(a); without classification and without abstract; title not checked by the International Searching Authority.</i></p>
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PLATELET-DERIVED GROWTH FACTOR C,  
DNA CODING THEREFOR, AND USES THEREOF

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10 BACKGROUND OF THE INVENTION

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This invention relates to growth factors for connective tissue cells, fibroblasts, myofibroblasts and glial cells and/or to growth factors for endothelial cells, and in particular to a novel platelet-derived growth factor/vascular endothelial growth factor-like growth factor, a polynucleotide sequence encoding the factor, and to pharmaceutical and diagnostic compositions and methods utilizing or derived from the factor.

In the developing embryo, the primary vascular network is established by *in situ* differentiation of mesodermal cells in a process called vasculogenesis. It is believed that all subsequent processes involving the generation of new vessels in the embryo and neovascularization in adults, are governed by the sprouting or splitting of new capillaries from the pre-existing vasculature in a process called angiogenesis (Pepper et al., *Enzyme & Protein*, 1996 49 138-162; Breier et al., *Dev. Dyn.* 1995 204 228-239; Risau, *Nature*, 1997 386 671-674). Angiogenesis is not only involved in embryonic development and normal tissue growth, repair, and regeneration, but is also involved in the female reproductive cycle, establishment and maintenance of pregnancy, and in repair of wounds and fractures. In addition to angiogenesis which takes place in the normal individual, angiogenic events are involved in a number of pathological processes, notably tumor growth and metastasis, and other conditions in which blood vessel proliferation, especially of the microvascular system, is increased, such as diabetic retinopathy, psoriasis and arthropathies. Inhibition of angiogenesis is useful in preventing or alleviating these pathological processes.

On the other hand, promotion of angiogenesis is desirable in situations where vascularization is to be established or

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extended, for example after tissue or organ transplantation, or to stimulate establishment of collateral circulation in tissue infarction or arterial stenosis, such als in coronary heart disease and thromboangitis obliterans.

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The angiogenic process is highly complex and involves the maintenance of the endothelial cells in the cell cycle, degradation of the extracellular matrix, migration and invasion of the surrounding tissue and finally, tube formation. The molecular mechanisms underlying the complex angiogenic processes are far from being understood.

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Because of the crucial role of angiogenesis in so many physiological and pathological processes, factors involved in the control of angiogenesis have been intensively investigated. A number of growth factors have been shown to be involved in the regulation of angiogenesis; these include fibroblast growth factors (FGFs), platelet-derived growth factor (PDGF), transforming growth factor alpha (TGF $\alpha$ ), and hepatocyte growth factor (HGF). See for example Folkman et al., J. Biol. Chem., 1992 267 10931-10934 for a review.

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It has been suggested that a particular family of endothelial cell-specific growth factors, the vascular endothelial growth factors (VEGFs), and their corresponding receptors is primarily responsible for stimulation of endothelial cell growth and differentiation, and for certain functions of the differentiated cells. These factors are members of the PDGF family, and appear to act primarily via endothelial receptor tyrosine kinases (RTKs).

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Nine different proteins have been identified in the PDGF family, namely two PDGFs (A and B), VEGF and six members that are closely related to VEGF. The six members closely related to VEGF are: VEGF-B, described in International Patent Application PCT/US96/02957 (WO 96/26736) and in U.S. Patents 5,840,693 and 5,607,918 by Ludwig Institute for Cancer

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Research and The University of Helsinki; VEGF-C, described in Jpukov et al., EMBO J., 1996 15 290-298 and Lee et al., Proc. Natl. Acad. Sci. USA, 1996 93 1988-1992; VEGF-D,) described in International Patent Application No. PCT/US97/14696 (WO 98/07832), and Achen et al., Proc. Natl. Acad. Sci. USA, 1998 95 548-553; the placenta growth factor (PlGF), described in Maglione et al., Proc. Natl. Acad. Sci. USA, 1991 88 9267-9271; VEGF2, described in International Patent Application No. PCT/US94/05291 (WO 95/24473) by Human Genome Sciences, Inc; and VEGF3, described in International Patent Application No. PCT/US95/07283 (WO 96/39421) by Human Genome Sciences, Inc. Each VEGF family member has between 30% and 45% amino acid sequence identity with VEGF. The VEGF family members share a VEGF homology domain which contains the six cysteine residues which form the cysteine knot motif. Functional characteristics of the VEGF family include varying degrees of mitogenicity for endothelial cells, induction of vascular permeability and angiogenic and lymphangiogenic properties.

Vascular endothelial growth factor (VEGF) is a homodimeric glycoprotein that has been isolated from several sources. VEGF shows highly specific mitogenic activity for endothelial cells. VEGF has important regulatory functions in the formation of new blood vessels during embryonic vasculogenesis and in angiogenesis during adult life (Carmeliet et al., Nature, 1996 380 435-439; Ferrara et al., Nature, 1996 380 439-442; reviewed in Ferrara and Davis-Smyth, Endocrine Rev., 1997 18 4-25). The significance of the role played by VEGF has been demonstrated in studies showing that inactivation of a single VEGF allele results in embryonic lethality due to failed development of the vasculature (Carmeliet et al., Nature, 1996 380 435-439; Ferrara et al., Nature, 1996 380 439-442). In addition VEGF has strong chemoattractant activity towards monocytes, can induce the

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10 plasminogen activator and the plasminogen activator inhibitor  
in endothelial cells, and can also induce microvascular  
permeability. Because of the latter activity, it is sometimes  
referred to as vascular permeability factor (VPF). The  
5 isolation and properties of VEGF have been reviewed; see  
Ferrara et al., J. Cellular Biochem., 1991 47 211-218 and  
15 Connolly, J. Cellular Biochem., 1991 47 219-223. Alternative  
mRNA splicing of a single VEGF gene gives rise to five  
isoforms of VEGF.

10 VEGF-B has similar angiogenic and other properties to  
20 those of VEGF, but is distributed and expressed in tissues  
differently from VEGF. In particular, VEGF-B is very strongly  
expressed in heart, and only weakly in lung, whereas the  
reverse is the case for VEGF. This suggests that VEGF and  
25 VEGF-B, despite the fact that they are co-expressed in many  
tissues, may have functional differences.

VEGF-B was isolated using a yeast co-hybrid interaction  
30 trap screening technique by screening for cellular proteins  
which might interact with cellular resinoid acid-binding  
protein type I (CRABP-I). Its isolation and characteristics  
20 are described in detail in PCT/US96/02957 and in Olofsson et  
al., Proc. Natl. Acad. Sci. USA, 1996, 93 2576-2581.

35 VEGF-C was isolated from conditioned media of the PC-3  
prostate adenocarcinoma cell line (CRL1435) by screening for  
25 ability of the medium to produce tyrosine phosphorylation of  
the endothelial cell-specific receptor tyrosine kinase VEGFR-3  
(Flt4), using cells transfected to express VEGFR-3. VEGF-C  
40 was purified using affinity chromatography with recombinant  
VEGFR-3, and was cloned from a PC-3 cDNA library. Its  
isolation and characteristics are described in detail in  
45 30 Joukov et al., EMBO J., 1996 15 290-298.

VEGF-D was isolated from a human breast cDNA library,  
50 commercially available from Clontech, by screening with an

expressed sequence tag obtained from a human cDNA library designated "Soares Breast 3NbHBst" as a hybridization probe (Achen et al., Proc. Natl. Acad. Sci. USA, 1998 95 548-553). Its isolation and characteristics are described in detail in International Patent Application No. PCT/US97/14696 (WO98/07832).

The VEGF-D gene is broadly expressed in the adult human, but is certainly not ubiquitously expressed. VEGF-D is strongly expressed in heart, lung and skeletal muscle. Intermediate levels of VEGF-D are expressed in spleen, ovary, small intestine and colon, and a lower expression occurs in kidney, pancreas, thymus, prostate and testis. No VEGF-D mRNA was detected in RNA from brain, placenta, liver or peripheral blood leukocytes.

PlGF was isolated from a term placenta cDNA library. Its isolation and characteristics are described in detail in Maglione et al., Proc. Natl. Acad. Sci. USA, 1991 88 9267-9271. Presently its biological function is not well understood.

VEGF2 was isolated from a highly tumorigenic, oestrogen-independent human breast cancer cell line. While this molecule is stated to have about 22% homology to PDGF and 30% homology to VEGF, the method of isolation of the gene encoding VEGF2 is unclear, and no characterization of the biological activity is disclosed.

VEGF3 was isolated from a cDNA library derived from colon tissue. VEGF3 is stated to have about 36% identity and 66% similarity to VEGF. The method of isolation of the gene encoding VEGF3 is unclear and no characterization of the biological activity is disclosed.

Similarity between two proteins is determined by comparing the amino acid sequence and conserved amino acid substitutions of one of the proteins to the sequence of the

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second protein, whereas identity is determined without including the conserved amino acid substitutions.

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PDGF/VEGF family members act primarily by binding to receptor tyrosine kinases. Five endothelial cell-specific receptor tyrosine kinases have been identified, namely VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1), VEGFR-3 (Flt4), Tie and Tek/Tie-2. All of these have the intrinsic tyrosine kinase activity which is necessary for signal transduction. The essential, specific role in vasculogenesis and angiogenesis of VEGFR-1, VEGFR-2, VEGFR-3, Tie and Tek/Tie-2 has been demonstrated by targeted mutations inactivating these receptors in mouse embryos.

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The only receptor tyrosine kinases known to bind VEGFs are VEGFR-1, VEGFR-2 and VEGFR-3. VEGFR-1 and VEGFR-2 bind VEGF with high affinity, and VEGFR-1 also binds VEGF-B and PlGF. VEGF-C has been shown to be the ligand for VEGFR-3, and it also activates VEGFR-2 (Joukov et al., The EMBO Journal, 1996 15 290-298). VEGF-D binds to both VEGFR-2 and VEGFR-3. A ligand for Tek/Tie-2 has been described in International Patent Application No. PCT/US95/12935 (WO 96/11269) by Regeneron Pharmaceuticals, Inc. The ligand for Tie has not yet been identified.

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Recently, a novel 130-135 kDa VEGF isoform specific receptor has been purified and cloned (Soker et al., Cell, 1998 92 735-745). The VEGF receptor was found to specifically bind the VEGF<sub>165</sub> isoform via the exon 7 encoded sequence, which shows weak affinity for heparin (Soker et al., Cell, 1998 92 735-745). Surprisingly, the receptor was shown to be identical to human neuropilin-1 (NP-1), a receptor involved in early stage neuromorphogenesis. PlGF-2 also appears to interact with NP-1 (Migdal et al., J. Biol. Chem., 1998 273 22272-22278).

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VEGFR-1, VEGFR-2 and VEGFR-3 are expressed differently by endothelial cells. Both VEGFR-1 and VEGFR-2 are expressed in blood vessel endothelia (Oelrichs et al., *Oncogene*, 1992 8 11-18; Kaipainen et al., *J. Exp. Med.*, 1993 178 2077-2088; Dumont et al., *Dev. Dyn.*, 1995 203 80-92; Fong et al., *Dev. Dyn.*, 1996 207 1-10) and VEGFR-3 is mostly expressed in the lymphatic endothelium of adult tissues (Kaipainen et al., *Proc. Natl. Acad. Sci. USA*, 1995 92 3566-3570). VEGFR-3 is also expressed in the blood vasculature surrounding tumors.

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Disruption of the VEGFR genes results in aberrant development of the vasculature leading to embryonic lethality around midgestation. Analysis of embryos carrying a completely inactivated VEGFR-1 gene suggests that this receptor is required for functional organization of the endothelium (Fong et al., *Nature*, 1995 376 66-70). However, deletion of the intracellular tyrosine kinase domain of VEGFR-1 generates viable mice with a normal vasculature (Hiratsuka et al., *Proc. Natl. Acad. Sci. USA* 1998 95 9349-9354). The reasons underlying these differences remain to be explained but suggest that receptor signalling via the tyrosine kinase is not required for the proper function of VEGFR-1. Analysis of homozygous mice with inactivated alleles of VEGFR-2 suggests that this receptor is required for endothelial cell proliferation, hematopoiesis and vasculogenesis (Shalaby et al., *Nature*, 1995 376 62-66; Shalaby et al., *Cell*, 1997 89 981-990). Inactivation of VEGFR-3 results in cardiovascular failure due to abnormal organization of the large vessels (Dumont et al. *Science*, 1998 282 946-949).

Although VEGFR-1 is mainly expressed in endothelial cells during development, it can also be found in hematopoietic precursor cells during early stages of embryogenesis (Fong et al., *Nature*, 1995 376 66-70). In adults, monocytes and

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macrophages also express this receptor (Barleon et al., Blood, 1996 87 3336-3343). In embryos, VEGFR-1 is expressed by most, if not all, vessels (Breier et al., Dev. Dyn., 1995 204 228-239; Fong et al., Dev. Dyn., 1996 207 1-10).

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The receptor VEGFR-3 is widely expressed on endothelial cells during early embryonic development but as embryogenesis proceeds becomes restricted to venous endothelium and then to the lymphatic endothelium (Kaipainen et al., Cancer Res., 1994 54 6571-6577; Kaipainen et al., Proc. Natl. Acad. Sci. USA, 1995 92 3566-3570). VEGFR-3 is expressed on lymphatic endothelial cells in adult tissues. This receptor is essential for vascular development during embryogenesis. Targeted inactivation of both copies of the VEGFR-3 gene in mice resulted in defective blood vessel formation characterized by abnormally organized large vessels with defective lumens, leading to fluid accumulation in the pericardial cavity and cardiovascular failure at post-coital day 9.5. On the basis of these findings it has been proposed that VEGFR-3 is required for the maturation of primary vascular networks into larger blood vessels. However, the role of VEGFR-3 in the development of the lymphatic vasculature could not be studied in these mice because the embryos died before the lymphatic system emerged. Nevertheless it is assumed that VEGFR-3 plays a role in development of the lymphatic vasculature and lymphangiogenesis given its specific expression in lymphatic endothelial cells during embryogenesis and adult life. This is supported by the finding that ectopic expression of VEGF-C, a ligand for VEGFR-3, in the skin of transgenic mice, resulted in lymphatic endothelial cell proliferation and vessel enlargement in the dermis. Furthermore this suggests that VEGF-C may have a primary function in lymphatic endothelium, and a secondary

function in angiogenesis and permeability regulation which is shared with VEGF (Joukov et al., EMBO J., 1996 15 290-298).

Some inhibitors of the VEGF/VEGF-receptor system have been shown to prevent tumor growth via an anti-angiogenic mechanism; see Kim et al., Nature, 1993 362 841-844 and Saleh et al., Cancer Res., 1996 56 393-401.

As mentioned above, the VEGF family of growth factors are members of the PDGF family. PDGF plays a important role in the growth and/or motility of connective tissue cells, fibroblasts, myofibroblasts and glial cells (Heldin et al., "Structure of platelet-derived growth factor: Implications for functional properties", Growth Factor, 1993 8 245-252). In adults, PDGF stimulates wound healing (Robson et al., Lancet, 1992 339 23-25). Structurally, PDGF isoforms are disulfide-bonded dimers of homologous A- and B-polypeptide chains, arranged as homodimers (PDGF-AA and PDGF-BB) or a heterodimer (PDGF-AB).

PDGF isoforms exert their effects on target cells by binding to two structurally related receptor tyrosine kinases (RTKs). The alpha-receptor binds both the A- and B-chains of PDGF, whereas the beta-receptor binds only the B-chain. These two receptors are expressed by many in vitro grown cell lines, and are mainly expressed by mesenchymal cells in vivo. The PDGFs regulate cell proliferation, cell survival and chemotaxis of many cell types in vitro (reviewed in Heldin et al., Biochim Biophys Acta., 1998 1378 F79-113). In vivo, they exert their effects in a paracrine mode since they often are expressed in epithelial (PDGF-A) or endothelial cells (PDGF-B) in close apposition to the PDGFR expressing mesenchyme. In tumor cells and in cell lines grown in vitro, coexpression of the PDGFs and the receptors generate autocrine loops which are important for cellular transformation (Betsholtz et al., Cell,

1984 39 447-57; Keating et al., J. R. Coll Surg Edinb., 1990  
35 172-4). Overexpression of the PDGFs have been observed in  
several pathological conditions, including malignancies,  
arteriosclerosis, and fibroproliferative diseases (reviewed  
in Heldin et al., The Molecular and Cellular Biology of Wound  
Repair, New York: Plenum Press, 1996, 249-273).

The importance of the PDGFs as regulators of cell  
proliferation and survival are well illustrated by recent gene  
targeting studies in mice that have shown distinct  
physiological roles for the PDGFs and their receptors despite  
the overlapping ligand specificities of the PDGFRs.  
Homozygous null mutations for either of the two PDGF ligands  
or the receptors are lethal. Approximately 50% of the  
homozygous PDGF-A deficient mice have an early lethal  
phenotype, while the surviving animals have a complex  
postnatal phenotype with lung emphysema due to improper  
alveolar septum formation because of a lack of alveolar  
myofibroblasts (Boström et al., Cell, 1996 85 863-873). The  
PDGF-A deficient mice also have a dermal phenotype  
characterized by thin dermis, misshapen hair follicles and  
thin hair (Karlsson et al., Development, 1999 126 2611-2).  
PDGF-A is also required for normal development of  
oligodendrocytes and subsequent myelination of the central  
nervous system (Fruttiger et al., Development, 1999 126  
457-67). The phenotype of PDGFR-alpha deficient mice is more  
severe with early embryonic death at E10, incomplete cephalic  
closure, impaired neural crest development, cardiovascular  
defects, skeletal defects, and odemas [Soriano et al.,  
Development, 1997 124 2691-70). The PDGF-B and PDGFR-beta  
deficient mice develop similar phenotypes that are  
characterized by renal, hematological and cardiovascular  
abnormalities (Levéen et al., Genes Dev., 1994 8 1875-1887;  
Soriano et al., Genes Dev., 1994 8 1888-96; Lindahl et al.,

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Science, 1997 277 242-5; Lindahl, Development, 1998 125 3313-2), where the renal and cardiovascular defects, at least in part, are due to the lack of proper recruitment of mural cells (vascular smooth muscle cells, pericytes or mesangial cells) to blood vessels (Levéen et al., Genes Dev., 1994 8 1875-1887; Lindahl et al., Science, 1997 277 242-5; Lindahl et al., Development, 1998 125 3313-2).

10 SUMMARY OF THE INVENTION

20 The invention generally provides an isolated novel growth factor which has the ability to stimulate and/or enhance proliferation or differentiation and/or growth and/or motility of cells expressing a PDGF-C receptor including, but not  
25 15 limited to, endothelial cells, connective tissue cells, myofibroblasts and glial cells, an isolated polynucleotide sequence encoding the novel growth factor, and compositions useful for diagnostic and/or therapeutic applications.

30 According to one aspect, the invention provides an isolated and purified nucleic acid molecule which comprises  
20 a polynucleotide sequence having at least 85% identity, more preferably at least 90%, and most preferably at least 95% identity to at least nucleotides 37-1071 of the sequence set out in Figure 1 (SEQ ID NO:2), at least nucleotides 6-956 of the sequence set out in Figure 3 (SEQ ID NO:3) or at least  
35 25 nucleotides 196 to 1233 of the sequence set out in Figure 5 (SEQ ID NO:6). The sequence of at least nucleotides 37-1071 of the sequence set out in Figure 1 (SEQ ID NO:2) or at least nucleotides 196 to 1233 of the sequence set out in Figure 5  
40 30 (SEQ ID NO:6) encodes a novel polypeptide, designated PDGF-C (formally designated "VEGF-F"), which is structurally homologous to PDGF-A, PDGF-B, VEGF, VEGF-B, VEGF-C and VEGF-D. In a preferred embodiment, the nucleic acid molecule is a cDNA

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which comprises at least nucleotides 37-1071 of the sequence set out in Figure 1 (SEQ ID NO:2), at least nucleotides 6-956 of the sequence set out in Figure 3 (SEQ ID NO:3) or at least nucleotides 196 to 1233 of the sequence set out in Figure 5 (SEQ ID NO:6). This aspect of the invention also encompasses DNA molecules having a sequence such that they hybridize under stringent conditions with at least nucleotides 37-1071 of the sequence set out in Figure 1 (SEQ ID NO:2), at least nucleotides 6-956 of the sequence set out in Figure 3 (SEQ ID NO:3) or at least nucleotides 196 to 1233 of the sequence set out in Figure 5 (SEQ ID NO:6) or fragments thereof.

According to a second aspect, the polypeptide of the invention has the ability to stimulate and/or enhance proliferation and/or differentiation and/or growth and/or motility of cells expressing a PDGF-C receptor including, but not limited to, endothelial cells, connective tissue cells, myofibroblasts and glial cells and comprises a sequence of amino acids corresponding to the amino acid sequence set out in Figure 2 (SEQ ID NO:3), Figure 4 (SEQ ID NO:5) or Figure 6 (SEQ ID NO:7), or a fragment or analog thereof which has the ability to stimulate and/or enhance proliferation and/or differentiation and/or growth and/or motility of cells expressing a PDGF-C receptor including, but not limited to, endothelial cells, connective tissue cells (such as fibroblasts), myofibroblasts and glial cells. Preferably the polypeptides have at least 85% identity, more preferably at least 90%, and most preferably at least 95% identity to the amino acid sequence of in Figure 2 (SEQ ID NO:3), Figure 4 (SEQ ID NO:5) or Figure 6 (SEQ ID NO:7), or a fragment or analog thereof having the biological activity of PDGF-C. A preferred fragment is a truncated form of PDGF-C comprising a portion of the PDGF/VEGF homology domain (PVHD) of PDGF-C. The minimal domain is residues 230-345. However, the domain

can extend towards the N terminus up to residue 164. Herein the PVHD is defined as truncated PDGF-C. The truncated PDGF-C is an activated form of PDGF-C.

As used in this application, percent sequence identity is determined by using the alignment tool of "MEGALIGN" from the Lasergene package (DNASTAR, Ltd. Abacus House, Manor Road, West Ealing, London W130AS United Kingdom) and using its preset conditions. The alignment is then refined manually, and the number of identities are estimated in the regions available for a comparison.

Preferably the polypeptide or the encoded polypeptide from a polynucleotide has the ability to stimulate one or more of proliferation, differentiation, motility, survival or vascular permeability of cells expressing a PDGF-C receptor including, but not limited to, vascular endothelial cells, lymphatic endothelial cells, connective tissue cells (such as fibroblasts), myofibroblasts and glial cells. Preferably the polypeptide or the encoded polypeptide from a polynucleotide has the ability to stimulate wound healing. PDGF-C can also have antagonistic effects on cells, but are included in the biological activities of PDGF-C. These abilities are referred to hereinafter as "biological activities of PDGF-C" and can be readily tested by methods known in the art.

As used herein, the term "PDGF-C" collectively refers to the polypeptides of Figure 2 (SEQ ID NO:3), Figure 4 (SEQ ID NO:5) or Figure 6 (SEQ ID NO:7), and fragments or analogs thereof which have the biological activity of PDGF-C as defined above, and to a polynucleotide which can code for PDGF-C, or a fragment or analog thereof having the biological activity of PDGF-C. The polynucleotide can be naked and/or in a vector or liposome.

In another preferred aspect, the invention provides a polypeptide possessing an amino acid sequence:

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PXCLLVXRCGGXCXCC (SEQ ID NO:1)

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which is unique to PDGF-C and differs from the other members of the PDGF/VEGF family of growth factors because of the insertion of the three amino acid residues (NCA) between the third and fourth cysteines (see Figure 9 - SEQ ID NOs:8-17).

Polypeptides comprising conservative substitutions, insertions, or deletions, but which still retain the biological activity of PDGF-C are clearly to be understood to be within the scope of the invention. Persons skilled in the art will be well aware of methods which can readily be used to generate such polypeptides, for example the use of site-directed mutagenesis, or specific enzymatic cleavage and ligation. The skilled person will also be aware that peptidomimetic compounds or compounds in which one or more amino acid residues are replaced by a non-naturally occurring amino acid or an amino acid analog may retain the required aspects of the biological activity of PDGF-C. Such compounds can readily be made and tested by methods known in the art, and are also within the scope of the invention.

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In addition, possible variant forms of the PDGF-C polypeptide which may result from alternative splicing, as are known to occur with VEGF and VEGF-B, and naturally-occurring allelic variants of the nucleic acid sequence encoding PDGF-C are encompassed within the scope of the invention. Allelic variants are well known in the art, and represent alternative forms or a nucleic acid sequence which comprise substitution, deletion or addition of one or more nucleotides, but which do not result in any substantial functional alteration of the encoded polypeptide.

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Such variant forms of PDGF-C can be prepared by targeting non-essential regions of the PDGF-C polypeptide for modification. These non-essential regions are expected to fall outside the strongly-conserved regions indicated in

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Figure 9 (SEQ ID NOS:8-17). In particular, the growth factors of the PDGF family, including VEGF, are dimeric, and VEGF, VEGF-B, VEGF-C, VEGF-D, PlGF, PDGF-A and PDGF-B show complete conservation of eight cysteine residues in the N-terminal domains, i.e. the PDGF/VEGF-like domains (Olofsson et al., Proc. Natl. Acad. Sci. USA, 1996 93 2576-2581; Joukov et al., EMBO J., 1996 15 290-298). These cysteines are thought to be involved in intra- and inter-molecular disulfide bonding. In addition there are further strongly, but not completely, conserved cysteine residues in the C-terminal domains. Loops 1, 2 and 3 of each subunit, which are formed by intra-molecular disulfide bonding, are involved in binding to the receptors for the PDGF/VEGF family of growth factors (Andersson et al., Growth Factors, 1995 12 159-164).

Persons skilled in the art thus are well aware that these cysteine residues should be preserved in any proposed variant form, and that the active sites present in loops 1, 2 and 3 also should be preserved. However, other regions of the molecule can be expected to be of lesser importance for biological function, and therefore offer suitable targets for modification. Modified polypeptides can readily be tested for their ability to show the biological activity of PDGF-C by routine activity assay procedures such as the fibroblast proliferation assay of Example 6.

It is contemplated that some modified PDGF-C polypeptides will have the ability to bind to PDGF-C receptors on cells including, but not limited to, endothelial cells, connective tissue cells, myofibroblasts and/or glial cells, but will be unable to stimulate cell proliferation, differentiation, migration, motility or survival or to induce vascular proliferation, connective tissue development or wound healing. These modified polypeptides are expected to be able to act as competitive or non-competitive inhibitors of the PDGF-C

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polypeptides and growth factors of the PDGF/VEGF family, and to be useful in situations where prevention or reduction of the PDGF-C polypeptide or PDGF/VEGF family growth factor action is desirable. Thus such receptor-binding but non-mitogenic, non-differentiation inducing, non-migration inducing, non-motility inducing, non-survival promoting, non-connective tissue development promoting, non-wound healing or non-vascular proliferation inducing variants of the PDGF-C polypeptide are also within the scope of the invention, and are referred to herein as "receptor-binding but otherwise inactive variant". Because PDGF-C forms a dimer in order to activate its only known receptor, it is contemplated that one monomer comprises the receptor-binding but otherwise inactive variant modified PDGF-C polypeptide and a second monomer comprises a wild-type PDGF-C or a wild-type growth factor of the PDGF/VEGF family. These dimers can bind to its corresponding receptor but cannot induce downstream signaling.

It is also contemplated that there are other modified PDGF-C polypeptides that can prevent binding of a wild-type PDGF-C or a wild-type growth factor of the PDGF/VEGF family to its corresponding receptor on cells including, but not limited to, endothelial cells, connective tissue cells (such as fibroblasts), myofibroblasts and/or glial cells. Thus these dimers will be unable to stimulate endothelial cell proliferation, differentiation, migration, survival, or induce vascular permeability, and/or stimulate proliferation and/or differentiation and/or motility of connective tissue cells, myofibroblasts or glial cells. These modified polypeptides are expected to be able to act as competitive or non-competitive inhibitors of the PDGF-C growth factor or a growth factor of the PDGF/VEGF family, and to be useful in situations where prevention or reduction of the PDGF-C growth factor or PDGF/VEGF family growth factor action is desirable. Such

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situations include the tissue remodeling that takes place during invasion of tumor cells into a normal cell population by primary or metastatic tumor formation. Thus such the PDGF-C or PDGF/VEGF family growth factor-binding but non-mitogenic, non-differentiation inducing, non-migration inducing, non-motility inducing, non-survival promoting, non-connective tissue promoting, non-wound healing or non-vascular proliferation inducing variants of the PDGF-C growth factor are also within the scope of the invention, and are referred to herein as "the PDGF-C growth factor-dimer forming but otherwise inactive or interfering variants".

An example of a PDGF-C growth factor-dimer forming but otherwise inactive or interfering variant is where the PDGF-C has a mutation which prevents cleavage of CUB domain from the protein. It is further contemplated that a PDGF-C growth factor-dimer forming but otherwise inactive or interfering variant could be made to comprise a monomer, preferably an activated monomer, of VEGF, VEGF-B, VEGF-C, VEGF-D, PDGF-C, PDGF-A, PDGF-B or PlGF linked to a CUB domain that has a mutation which prevents cleavage of CUB domain from the protein. Dimers formed with the above mentioned PDGF-C growth factor-dimer forming but otherwise inactive or interfering variants and the monomers linked to the mutant CUB domain would be unable to bind to their corresponding receptors.

A variation on this contemplation would be to insert a proteolytic site between an activated monomer of VEGF, VEGF-B, VEGF-C, VEGF-D, PDGF-C, PDGF-A, PDGF-B or PlGF and the mutant CUB domain linkage which is dimerized to an activated monomer of VEGF, VEGF-B, VEGF-C, VEGF-D, PDGF-C, PDGF-A, PDGF-B or PlGF. An addition of the specific protease(s) for this proteolytic site would cleave the CUB domain and thereby release an activated dimer that can then bind to its

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corresponding receptor. In this way, a controlled release of  
an activated dimer is made possible.

10 According to a third aspect, the invention provides a  
purified and isolated nucleic acid encoding a polypeptide or  
5 polypeptide fragment of the invention as defined above. The  
nucleic acid may be DNA, genomic DNA, cDNA or RNA, and may be  
15 single-stranded or double stranded. The nucleic acid may be  
isolated from a cell or tissue source, or of recombinant or  
synthetic origin. Because of the degeneracy of the genetic  
20 code, the person skilled in the art will appreciate that many  
such coding sequences are possible, where each sequence  
encodes the amino acid sequence shown in Figure 2 (SEQ ID  
NO:3), Figure 4 (SEQ ID NO:5) or Figure 6 (SEQ ID NO:7), a  
25 bioactive fragment or analog thereof, a receptor-binding but  
otherwise inactive or partially inactive variant thereof or  
a PDGF-C-dimer forming but otherwise inactive or interfering  
variants thereof.

30 A fourth aspect of the invention provides vectors  
comprising the cDNA of the invention or a nucleic acid  
20 molecule according to the third aspect of the invention, and  
host cells transformed or transfected with nucleic acids  
molecules or vectors of the invention. These may be  
35 eukaryotic or prokaryotic in origin. These cells are  
particularly suitable for expression of the polypeptide of the  
invention, and include insect cells such as Sf9 cells,  
40 obtainable from the American Type Culture Collection  
(ATCC SRL-171), transformed with a baculovirus vector, and the  
human embryo kidney cell line 293-EBNA transfected by a  
suitable expression plasmid. Preferred vectors of the  
45 30 invention are expression vectors in which a nucleic acid  
according to the invention is operatively connected to one or  
more appropriate promoters and/or other control sequences,  
such that appropriate host cells transformed or transfected

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10 with the vectors are capable of expressing the polypeptide of the invention. Other preferred vectors are those suitable for transfection of mammalian cells, or for gene therapy, such as adenoviral-, vaccinia- or retroviral-based vectors or liposomes. A variety of such vectors is known in the art.

15 The invention also provides a method of making a vector capable of expressing a polypeptide encoded by a nucleic acid according to the invention, comprising the steps of operatively connecting the nucleic acid to one or more appropriate promoters and/or other control sequences, as described above.

20 The invention further provides a method of making a polypeptide according to the invention, comprising the steps of expressing a nucleic acid or vector of the invention in a host cell, and isolating the polypeptide from the host cell or from the host cell's growth medium.

25 In yet a further aspect, the invention provides an antibody specifically reactive with a polypeptide of the invention or a fragment of the polypeptide. This aspect of the invention includes antibodies specific for the variant forms, immunoreactive fragments, analogs and recombinants of PDGF-C. Such antibodies are useful as inhibitors or agonists of PDGF-C and as diagnostic agents for detecting and quantifying PDGF-C. Polyclonal or monoclonal antibodies may be used. Monoclonal and polyclonal antibodies can be raised against polypeptides of the invention or fragment or analog thereof using standard methods in the art. In addition the polypeptide can be linked to an epitope tag, such as the FLAG<sup>®</sup> octapeptide (Sigma, St. Louis, MO), to assist in affinity purification. For some purposes, for example where a monoclonal antibody is to be used to inhibit effects of PDGF-C in a clinical situation, it may be desirable to use humanized or chimeric monoclonal antibodies. Such antibodies may be

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further modified by addition of cytotoxic or cytostatic drugs. Methods for producing these, including recombinant DNA methods, are also well known in the art.

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This aspect of the invention also includes an antibody which recognizes PDGF-C and is suitably labeled.

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Polypeptides or antibodies according to the invention may be labeled with a detectable label, and utilized for diagnostic purposes. Similarly, the thus-labeled polypeptide of the invention may be used to identify its corresponding receptor *in situ*. The polypeptide or antibody may be covalently or non-covalently coupled to a suitable supermagnetic, paramagnetic, electron dense, ecogenic or radioactive agent for imaging. For use in diagnostic assays, radioactive or non-radioactive labels may be used. Examples of radioactive labels include a radioactive atom or group, such as  $^{125}\text{I}$  or  $^{32}\text{P}$ . Examples of non-radioactive labels include enzymatic labels, such as horseradish peroxidase or fluorimetric labels, such as fluorescein-5-isothiocyanate (FITC). Labeling may be direct or indirect, covalent or non-covalent.

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Clinical applications of the invention include diagnostic applications, acceleration of angiogenesis in tissue or organ transplantation, or stimulation of wound healing, or connective tissue development, or to establish collateral circulation in tissue infarction or arterial stenosis, such as coronary artery disease, and inhibition of angiogenesis in the treatment of cancer or of diabetic retinopathy and inhibition of tissue remodeling that takes place during invasion of tumor cells into a normal cell population by primary or metastatic tumor formation. Quantitation of PDGF-C in cancer biopsy specimens may be useful as an indicator of future metastatic risk.

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PDGF-C may also be relevant to a variety of lung

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conditions. PDGF-C assays could be used in the diagnosis of various lung disorders. PDGF-C could also be used in the treatment of lung disorders to improve blood circulation in the lung and/or gaseous exchange between the lungs and the blood stream. Similarly, PDGF-C could be used to improve blood circulation to the heart and O<sub>2</sub> gas permeability in cases of cardiac insufficiency. In a like manner, PDGF-C could be used to improve blood flow and gaseous exchange in chronic obstructive airway diseases.

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Thus the invention provides a method of stimulation of angiogenesis, lymphangiogenesis, neovascularization, connective tissue development and/or wound healing in a mammal in need of such treatment, comprising the step of administering an effective dose of PDGF-C, or a fragment or an analog thereof which has the biological activity of PDGF-C to the mammal. Optionally the PDGF-C, or fragment or analog thereof may be administered together with, or in conjunction with, one or more of VEGF, VEGF-B, VEGF-C, VEGF-D, PlGF, PDGF-A, PDGF-B, FGF and/or heparin.

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Conversely, PDGF-C antagonists (e.g. antibodies and/or competitive or noncompetitive inhibitors of binding of PDGF-C in both dimer formation and receptor binding) could be used to treat conditions, such as congestive heart failure, involving accumulation of fluid in, for example, the lung resulting from increases in vascular permeability, by exerting an offsetting effect on vascular permeability in order to counteract the fluid accumulation. PDGF-C can also be used to treat fibrotic conditions including those found in the lung, kidney and liver. Administrations of PDGF-C could be used to treat malabsorptive syndromes in the intestinal tract, liver or kidneys as a result of its blood circulation increasing and vascular permeability increasing activities.

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Thus, the invention provides a method of inhibiting angiogenesis, lymphangiogenesis, neovascularization, connective tissue development and/or wound healing in a mammal in need of such treatment, comprising the step of administering an effective amount of an antagonist of PDGF-C to the mammal. The antagonist may be any agent that prevents the action of PDGF-C, either by preventing the binding of PDGF-C to its corresponding receptor on the target cell, or by preventing activation of the receptor, such as using receptor-binding PDGF-C variants. Suitable antagonists include, but are not limited to, antibodies directed against PDGF-C; competitive or non-competitive inhibitors of binding of PDGF-C to the PDGF-C receptor(s), such as the receptor-binding or PDGF-C dimer-forming but non-mitogenic PDGF-C variants referred to above; compounds that bind to PDGF-C and/or modify or antagonize its function, and anti-sense nucleotide sequences as described below.

A method is provided for determining agents that bind to an activated truncated form of PDGF-C. The method comprises contacting an activated truncated form of PDGF-C with a test agent and monitoring binding by any suitable means. Agents can include both compounds and other proteins.

The invention provides a screening system for discovering agents that bind an activated truncated form of PDGF-C. The screening system comprises preparing an activated truncated form of PDGF-C, exposing the activated truncated form of PDGF-C to a test agent, and quantifying the binding of said agent to the activated truncated form of PDGF-C by any suitable means. This screening system can also be used to identify agents which inhibit the proteolytic cleavage of the full length PDGF-C protein and thereby prevent the release of the activated truncated form of PDGF-C. For this use, the full length PDGF-C must be prepared.



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Use of this screen system provides a means to determine compounds that may alter the biological function of PDGF-C. This screening method may be adapted to large-scale, automated procedures such as a PANDEX® (Baxter-Dade Diagnostics) system, allowing for efficient high-volume screening of potential therapeutic agents.

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For this screening system, an activated truncated form of PDGF-C or full length PDGF-C is prepared as described herein, preferably using recombinant DNA technology. A test agent, e.g. a compound or protein, is introduced into a reaction vessel containing the activated truncated form of or full length PDGF-C. Binding of the test agent to the activated truncated form of or full length PDGF-C is determined by any suitable means which include, but is not limited to, radioactively- or chemically-labeling the test agent. Binding of the activated truncated form of or full length PDGF-C may also be carried out by a method disclosed in U.S. Patent 5,585,277, which is incorporated by reference. In this method, binding of the test agent to the activated truncated form of or full length PDGF-C is assessed by monitoring the ratio of folded protein to unfolded protein. Examples of this monitoring can include, but are not limited to, monitoring the sensitivity of the activated truncated form of or full length PDGF-C to a protease, or amenability to binding of the protein by a specific antibody against the folded state of the protein.

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Those of skill in the art will recognize that  $IC_{50}$  values are dependent on the selectivity of the agent tested. For example, an agent with an  $IC_{50}$  which is less than 10 nM is generally considered an excellent candidate for drug therapy. However, an agent which has a lower affinity, but is selective for a particular target, may be an even better candidate. Those skilled in the art will recognize that any information

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10 regarding the binding potential, inhibitory activity or selectivity of a particular agent is useful toward the development of pharmaceutical products.

5 Where a PDGF-C or a PDGF-C antagonist is to be used for therapeutic purposes, the dose(s) and route of administration will depend upon the nature of the patient and condition to be treated, and will be at the discretion of the attending physician or veterinarian. Suitable routes include oral, 15 subcutaneous, intramuscular, intraperitoneal or intravenous injection, parenteral, topical application, implants etc. 20 Topical application of PDGF-C may be used in a manner analogous to VEGF. For example, where used for wound healing or other use in which enhanced angiogenesis is advantageous, an effective amount of the truncated active form of PDGF-C is 25 administered to an organism in need thereof in a dose between about 0.1 and 1000 µg/kg body weight.

30 The PDGF-C or a PDGF-C antagonist may be employed in combination with a suitable pharmaceutical carrier. The resulting compositions comprise a therapeutically effective amount of PDGF-C or a PDGF-C antagonist, and a 35 pharmaceutically acceptable non-toxic salt thereof, and a pharmaceutically acceptable solid or liquid carrier or adjuvant. Examples of such a carrier or adjuvant include, but are not limited to, saline, buffered saline, Ringer's 40 solution, mineral oil, talc, corn starch, gelatin, lactose, sucrose, microcrystalline cellulose, kaolin, mannitol, dicalcium phosphate, sodium chloride, alginic acid, dextrose, water, glycerol, ethanol, thickeners, stabilizers, suspending agents and combinations thereof. Such compositions may be in 45 30 the form of solutions, suspensions, tablets, capsules, creams, salves, elixirs, syrups, wafers, ointments or other conventional forms. The formulation to suit the mode of administration. Compositions which comprise PDGF-C may 50

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optionally further comprise one or more of PDGF-A, PDGF-B, VEGF, VEGF-B, VEGF-C, VEGF-D, PlGF and/or heparin. Compositions comprising PDGF-C will contain from about 0.1% to 90% by weight of the active compound(s), and most generally from about 10% to 30%.

For intramuscular preparations, a sterile formulation, preferably a suitable soluble salt form of the truncated active form of PDGF-C, such as hydrochloride salt, can be dissolved and administered in a pharmaceutical diluent such as pyrogen-free water (distilled), physiological saline or 5% glucose solution. A suitable insoluble form of the compound may be prepared and administered as a suspension in an aqueous base or a pharmaceutically acceptable oil base, e.g. an ester of a long chain fatty acid such as ethyl oleate.

According to yet a further aspect, the invention provides diagnostic/prognostic devices typically in the form of test kits. For example, in one embodiment of the invention there is provided a diagnostic/prognostic test kit comprising an antibody to PDGF-C and a means for detecting, and more preferably evaluating, binding between the antibody and PDGF-C. In one preferred embodiment of the diagnostic/prognostic device according to the invention, a second antibody (the secondary antibody) directed against antibodies of the same isotype and animal source of the antibody directed against PDGF-C (the primary antibody) is provided. The secondary antibody is coupled to a detectable label, and then either an unlabeled primary antibody or PDGF-C is substrate-bound so that the PDGF-C/primary antibody interaction can be established by determining the amount of label bound to the substrate following binding between the primary antibody and PDGF-C and the subsequent binding of the labeled secondary antibody to the primary antibody. In a particularly preferred embodiment of the invention, the diagnostic/prognostic device

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may be provided as a conventional enzyme-linked immunosorbent assay (ELISA) kit.

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In another alternative embodiment, a diagnostic/prognostic device may comprise polymerase chain reaction means for establishing sequence differences of a PDGF-C of a test individual and comparing this sequence structure with that disclosed in this application in order to detect any abnormalities, with a view to establishing whether any aberrations in PDGF-C expression are related to a given disease condition.

In addition, a diagnostic/prognostic device may comprise a restriction length polymorphism (RFLP) generating means utilizing restriction enzymes and genomic DNA from a test individual to generate a pattern of DNA bands on a gel and comparing this pattern with that disclosed in this application in order to detect any abnormalities, with a view to establishing whether any aberrations in PDGF-C expression are related to a given disease condition.

In accordance with a further aspect, the invention relates to a method of detecting aberrations in PDGF-C gene in a test subject which may be associated with a disease condition in the test subject. This method comprises providing a DNA or RNA sample from said test subject; contacting the DNA sample or RNA with a set of primers specific to PDGF-C DNA operatively coupled to a polymerase and selectively amplifying PDGF-C DNA from the sample by polymerase chain reaction, and comparing the nucleotide sequence of the amplified PDGF-C DNA from the sample with the nucleotide sequences shown in Figure 1 (SEQ ID NO:2) or Figure 3 (SEQ ID NO:5). The invention also includes the provision of a test kit comprising a pair of primers specific to PDGF-C DNA operatively coupled to a polymerase, whereby said

polymerase is enabled to selectively amplify PDGF-C DNA from a DNA sample.

The invention also provides a method of detecting PDGF-C in a biological sample, comprising the step of contacting the sample with a reagent capable of binding PDGF-C, and detecting the binding. Preferably the reagent capable of binding PDGF-C is an antibody directed against PDGF-C, particularly preferably a monoclonal antibody. In a preferred embodiment the binding and/or extent of binding is detected by means of a detectable label; suitable labels are discussed above.

In another aspect, the invention relates to a protein dimer comprising the PDGF-C polypeptide, particularly a disulfide-linked dimer. The protein dimers of the invention include both homodimers of PDGF-C polypeptide and heterodimers of PDGF-C and VEGF, VEGF-B, VEGF-C, VEGF-D, PlGF, PDGF-A or PDGF-B.

According to a yet further aspect of the invention there is provided a method for isolation of PDGF-C comprising the step of exposing a cell which expresses PDGF-C to heparin to facilitate release of PDGF-C from the cell, and purifying the thus-released PDGF-C.

Another aspect of the invention involves providing a vector comprising an anti-sense nucleotide sequence which is complementary to at least a part of a DNA sequence which encodes PDGF-C or a fragment or analog thereof that has the biological activity of PDGF-C. In addition the anti-sense nucleotide sequence can be to the promoter region of the PDGF-C gene or other non-coding region of the gene which may be used to inhibit, or at least mitigate, PDGF-C expression.

According to a yet further aspect of the invention such a vector comprising an anti-sense sequence may be used to inhibit, or at least mitigate, PDGF-C expression. The use of a vector of this type to inhibit PDGF-C expression is favored

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in instances where PDGF-C expression is associated with a disease, for example where tumors produce PDGF-C in order to provide for angiogenesis, or tissue remodeling that takes place during invasion of tumor cells into a normal cell population by primary or metastatic tumor formation. Transformation of such tumor cells with a vector containing an anti-sense nucleotide sequence would inhibit or retard growth of the tumor or tissue remodeling.

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Another aspect of the invention relates to the discovery that the full length PDGF-C protein is likely to be a latent growth factor that needs to be activated by proteolytic processing to release an active PDGF/VEGF homology domain. A putative proteolytic site is found in residues 231-234 in the full length protein, residues -RKSR-. This is a dibasic motif. This site is structurally conserved in the mouse PDGF-C. The -RKSR- putative proteolytic site is also found in PDGF-A, PDGF-B, VEGF-C and VEGF-D. In these four proteins, the putative proteolytic site is also found just before the minimal domain for the PDGF/VEGF homology domain. Together these facts indicate that this is the proteolytic site.

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Preferred proteases include, but are not limited, to plasmin, Factor X and enterokinase. The N-terminal CUB domain may function as an inhibitory domain which might be used to keep PDGF-C in a latent form in some extracellular compartment and which is removed by limited proteolysis when PDGF-C is needed.

According to this aspect of the invention, a method is provided for producing an activated truncated form of PDGF-C or for regulating receptor-binding specificity of PDGF-C. These methods comprise the steps of expressing an expression vector comprising a polynucleotide encoding a polypeptide having the biological activity of PDGF-C and supplying a proteolytic amount of at least one enzyme for processing the

expressed polypeptide to generate the activated truncated form of PDGF-C.

This aspect also includes a method for selectively activating a polypeptide having a growth factor activity. This method comprises the step expressing an expression vector comprising a polynucleotide encoding a polypeptide having a growth factor activity, a CUB domain and a proteolytic site between the polypeptide and the CUB domain, and supplying a proteolytic amount of at least one enzyme for processing the expressed polypeptide to generate the activated polypeptide having a growth factor activity.

In addition, this aspect includes the isolation of a nucleic acid molecule which codes for a polypeptide having the biological activity of PDGF-C and a polypeptide thereof which comprises a proteolytic site having the amino acid sequence RKSR or a structurally conserved amino acid sequence thereof.

Also this aspect includes an isolated dimer comprising an activated monomer of PDGF-C and an activated monomer of VEGF, VEGF-B, VEGF-C, VEGF-D, PDGF-C, PDGF-A, PDGF-B or PlGF linked to a CUB domain, or alternatively, an activated monomer of VEGF, VEGF-B, VEGF-C, VEGF-D, PDGF-C, PDGF-A, PDGF-B or PlGF and an activated monomer of PDGF-C linked to a CUB domain. The isolated dimer may or may not include a proteolytic site between the activator monomer and the CUB domain linkage.

Polynucleotides of the invention such as those described above, fragments of those polynucleotides, and variants of those polynucleotides with sufficient similarity to the non-coding strand of those polynucleotides to hybridize thereto under stringent conditions all are useful for identifying, purifying, and isolating polynucleotides encoding other, non-human, mammalian forms of PDGF-C. Thus, such polynucleotide fragments and variants are intended as aspects of the

invention. Exemplary stringent hybridization conditions are as follows: hybridization at 42°C in 5X SSC, 20 mM NaPO<sub>4</sub>, pH 6.8, 50% formamide; and washing at 42°C in 0.2X SSC. Those skilled in the art understand that it is desirable to vary these conditions empirically based on the length and the GC nucleotide base content of the sequences to be hybridized, and that formulas for determining such variation exist. See for example Sambrook et al, "Molecular Cloning: A Laboratory Manual", Second Edition, pages 9.47-9.51, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory (1989).

Moreover, purified and isolated polynucleotides encoding other, non-human, mammalian PDGF-C forms also are aspects of the invention, as are the polypeptides encoded thereby and antibodies that are specifically immunoreactive with the non-human PDGF-C variants. Thus, the invention includes a purified and isolated mammalian PDGF-C polypeptide and also a purified and isolated polynucleotide encoding such a polypeptide.

It will be clearly understood that nucleic acids and polypeptides of the invention may be prepared by synthetic means or by recombinant means, or may be purified from natural sources.

It will be clearly understood that for the purposes of this specification the word "comprising" means "included but not limited to". The corresponding meaning applies to the word "comprises".

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 (SEQ ID NO:2) shows the complete nucleotide sequence of cDNA encoding a human PDGF-C (hPDGF-C) (2108 bp);

Figure 2 (SEQ ID NO:3) shows the deduced amino acid sequence of full-length hPDGF-C which consists of 345 amino



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acid residues (the translated part of the cDNA corresponds to nucleotides 37 to 1071 of Figure 1);

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Figure 3 (SEQ ID NO:4) shows a cDNA sequence encoding a fragment of human PDGF-C (hPDGF-C) (1536 bp);

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Figure 4 (SEQ ID NO:5) shows a deduced amino acid sequence of a fragment of hPDGF-C (translation of nucleotides 3 to 956 of the nucleotide sequence of Figure 3);

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Figure 5 (SEQ ID NO:6) shows a nucleotide sequence of a murine PDGF-C (mPDGF-C) cDNA;

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Figure 6 (SEQ ID NO:7) shows the deduced amino acid sequence of a fragment of mPDGF-C (the translated part of the cDNA corresponds to nucleotides 196 to 1233 of Figure 5);

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Figure 7 shows a comparative sequence alignment of the hPDGF-C amino acid sequence of Figure 2 (SEQ ID NO:3) with the mPDGF-C amino acid sequence of Figure 6 (SEQ ID NO:7);

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Figure 8 shows a schematic structure of mPDGF-C with a signal sequence (striped box), a N-terminal C1r/C1s/embryonic sea urchin protein Uegf/bone morphogenetic protein 1 (CUB) domain and the C-terminal PDGF/VEGF homology domain (open boxes);

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Figure 9 shows a comparative sequence alignment of the PDGF/VEGF-homology domains in human and mouse PDGF-C with other members of the VEGF/PDGF family of growth factors (SEQ ID NOs:8-17, respectively);

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Figure 10 shows a phylogenetic tree of several growth factors belonging to the VEGF/PDGF family;

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Figure 11 provides the amino acid sequence alignment of the CUB domain present in human and mouse PDGF-Cs (SEQ ID NOs:18 and 19, respectively) and other CUB domains present in human bone morphogenic protein-1 (hBMP-1, 3 CUB domains CUB1-3) (SEQ ID NOs:20-22, respectively) and in human neuropilin-1 (2 CUB domains) (SEQ ID NOs:23 and 24, respectively);

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Figure 12 shows a Northern blot analysis of the expression of PDGF-C transcripts in several human tissues;

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Figure 13 shows the regulation of PDGF-C mRNA expression by hypoxia; and

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Figure 14 shows the expression of PDGF-C in human tumor cell lines.

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Figure 15 shows the results of immunoblot detection of full length human PDGF-C in transfected COS-1 cells.

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Figure 16 shows isolation and partial characterization of full length PDGF-C.

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Figure 17 shows isolation and partial characterization of a truncated form of human PDGF-C containing the PDGF/VEGF homology domain only.

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Figure 18 provides a standard curve for the binding of labeled PDGF-BB homodimers to PAE-1 cells expressing PDGF alpha receptor.

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Figure 19 provides a graphic representation of the inhibition of binding of labeled PDGF-BB to PAE-1 cells expressing PDGF alpha receptor by increasing amounts of purified full length and truncated PDGF-CC proteins.

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Figure 20 shows the effects of the full length and truncated PDGF-CC homodimers on the phosphorylation of PDGF alpha-receptor.

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Figure 21 shows the mitogenic activities of the full length and truncated PDGF-CC homodimers on fibroblasts.

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Figure 22 graphically presents the results of the binding assay of truncated PDGF-C to the PDGF receptors.

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Figure 23 shows the immunoblot of the undigested full length PDGF-C protein and the plasmin-generated 26-28 kDa species.

Figure 24 graphically presents the results of the competitive binding assay of full-length PDGF-C and truncated PDGF-C for PDGFR-alpha receptors.

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Figure 25 shows the analyses by SDS-PAGE of the human PDGF-C CUB domain under reducing and non-reducing conditions.

Figures 26A-26V show PDGF-C expression in the developing mouse embryo.

Figures 27A-27F show PDGF-C, PDGF-A and PDGFR-alpha expression in the developing kidney.

Figures 28A-28F show histology of E 16.5 kidneys from wildtype (Figures 28A and 28C), PDGFR-alpha -/- (Figures 28B and 28F, PDGF-A -/- (Figure 28D) and PDGF-A/PDGF-B double -/- (Figure 28E) kidneys.

#### DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Figure 1 (SEQ ID NO:2) shows the complete nucleotide sequence of cDNA encoding a human PDGF-C (hPDGF-C) (2108 bp), which is a new member of the VEGF/PDGF family. A clone #4 (see Figures 3 and 4 - SEQ ID NOs:4 and 5) encoding hPDGF-C was not full length and lacked approximately 80 base pairs of coding sequence when compared to the mouse protein (corresponding to 27 amino acids). Additional cDNA clones were isolated from a human fetal lung cDNA library to obtain an insert which included this missing sequence. Clone #10 had a longer insert than clone #4. The insert of clone #10 was sequenced in the 5' region and it was found to contain the missing sequence. Clone #10 was found to include the full sequence of human PDGF-C. Some 5'-untranslated sequence, the translated part of the cDNA encoding human PDGF-C and some 3'-untranslated nucleotide sequence are shown in Figure 1 (SEQ ID NO:2). A stop codon in frame is located 21 bp upstream of the initiation ATG (the initiation ATG is underlined in Figure 1).

Work to isolate this new human PDGF/VEGF began after a search of the expressed sequence tag (EST) database, dbEST,

at the National Center for Biotechnology Information (NCBI) in Washington, DC, identified a human EST sequence (W21436) which appears to encode part of the human homolog of the mouse PDGF-C. Based on the human EST sequence, two oligonucleotides were designed:

5'-GAA GTT GAG GAA CCC AGT G-3' forward (SEQ ID NO:25)

5'-CTT GCC AAG AAG TTG CCA AG-3' reverse (SEQ ID NO:26).

These oligonucleotides were used to amplify by polymerase chain reaction (PCR) a polynucleotide of 348 bps from a Human Fetal Lung 5'-STRETCH PLUS  $\lambda$ gt10 cDNA library, which was obtained commercially from Clontech. The PCR product was cloned into the pCR 2.1-vector of the Original TA Cloning Kit (Invitrogen). Subsequently, the 348 bps cloned PCR product was used to construct a hPDGF-C probe according to standard techniques.

$10^6$  lambda-clones of the Human Fetal Lung 5'-STRETCH PLUS  $\lambda$ gt10 cDNA Library (Clontech) were screened with the hPDGF-C probe according to standard procedures. Among several positive clones, one, clone #4 was analyzed more carefully and the nucleotide sequence of its insert was determined according to standard procedures using internal and vector oligonucleotides. The insert of clone #4 contains a partial nucleotide sequence of the cDNA encoding the full length human PDGF-C (hPDGF-C). The nucleotide sequence (1536 bp) of the clone #4 insert is shown in Figure 3 (SEQ ID NO:4). The translated portion of this cDNA includes nucleotides 6 to 956. The deduced amino acid sequence of the translated portion of the insert is illustrated in Figure 4 (SEQ ID NO:5). A polypeptide of this deduced amino acid sequence would lack the first 28 amino acid residues found in the full length hPDGF-C polypeptide. However, this polypeptide includes a proteolytic fragment which is sufficient to activate the PDGF alpha

receptors. It should be noted that the first glycine (Gly) of SEQ ID NO:5 is not found in the full length hPDGF-C.

A mouse EST sequence (AI020581) was identified in a database search of the dbEST database at the NCBI in Washington, DC, which appears to encode part of a new mouse PDGF, PDGF-C. Large parts of the mouse cDNA was obtained by PCR amplification using DNA from a mouse embryo  $\lambda$ gt10 cDNA library as the template. To amplify the 3' end of the cDNA, a sense primer derived from the mouse EST sequence was used (the sequence of this primer was 5'-CTT CAG TAC CTT GGA AGA G, primer 1 (SEQ ID NO:27)). To amplify the 5' end of the cDNA, an antisense primer derived from the mouse EST was used (the sequence of this primer was 5'-CGC TTG ACC AGG AGA CAA C, primer 2 (SEQ ID NO:28)). The  $\lambda$ gt10 vector primers were sense 5'-ACG TGA ATT CAG CAA GTT CAG CCT GGT TAA (primer 3 (SEQ ID NO:29)) and antisense 5'-ACG TGG ATC CTG AGT ATT TCT TCC AGG GTA (primer 4 (SEQ ID NO:30)). Combinations of the vector primers and the internal primers obtained from the mouse EST were used in standard PCR reactions. The sizes of the amplified fragments were approx. 750 bp (3'-fragment) and 800 bp (5'-fragment), respectively. These fragments were cloned into the pCR 2.1 vector and subjected to nucleotide sequences analysis using vector primers and internal primers. Since these fragments did not contain the full length sequence of mPDGF-C, a mouse liver ZAP cDNA library was screened using standard conditions. A 261 bp  $^{32}$ P-labeled PCR fragment was generated for use as a probe using primers 1 and 2 and using DNA from the mouse embryo  $\lambda$ gt10 library as the template (see above). Several positive plaques were purified and the nucleotide sequence of the inserts were obtained following subcloning into pBluescript. Vector specific primers and internal primers were used. By combining the nucleotide sequence information of the generated PCR clones and the

isolated clone, the full length amino acid sequence of mPDGF-C could be deduced (see Figure 6) (SEQ ID NO:7).

Figure 7 shows a comparative sequence alignment of the mouse and human amino acid sequences of PDGF-C (SEQ ID NOS:6 and 2, respectively). The alignment shows that human and mouse PDGF-Cs display an identity of about 87% with 45 amino acid replacements found among the 345 residues of the full length proteins. Almost all of the observed amino acid replacements are conservative in nature. The predicted cleavage site in mPDGF-C for the signal peptidase is between residues G19 and T20. This would generate a secreted mouse peptide of 326 amino acid residues.

Figure 8 provides a schematic domain structure of mouse PDGF-C with a signal sequence (striped box), a N-terminal CUB domain and the C-terminal PDGF/VEGF-homology domain (open boxes). The amino acid sequences denoted by the lines have no obvious similarities to CUB domains or to VEGF-homology domains.

The high sequence identity suggests that human and mouse PDGF-C have an almost identical domain structure. Amino acid sequence comparisons revealed that both mouse and human PDGF-C display a novel domain structure. Apart from the PDGF/VEGF-homology domain located in the C-terminal region in both proteins (residues 164 to 345), the N-terminal region in both PDGF-Cs have a domain referred to as a CUB domain (Bork and Beckmann, J. Mol. Biol., 1993 231, 539-545). This domain of about 110 amino acids (amino acid residues 50-160) was originally identified in complement factors C1r/C1s, but has recently been identified in several other extracellular proteins including signaling molecules such as bone morphogenic protein 1 (BMP-1) (Wozney et al., Science, 1988 242, 1528-1534) as well as in several receptor molecules such

as neuropilin-1 (NP-1) (Soker et al., Cell, 1998 92 735-745). The functional roles of CUB domains are not clear but it may participate in protein-protein interactions or in interactions with carbohydrates including heparin sulfate proteoglycans.

Figure 9 shows the amino acid sequence alignment of the C-terminal PDGF/VEGF-homology domains of human and mouse PDGF-Cs with the C-terminal PDGF/VEGF-homology domains of PDGF/VEGF family members, VEGF<sub>165</sub>, PlGF-2, VEGF-B<sub>167</sub>, Pox Orf VEGF, VEGF-C, VEGF-D, PDGF-A and PDGF-B (SEQ ID NOs:8-17). Some of the amino acid sequences in the N- and C-terminal regions in VEGF-C and VEGF-D have been deleted in this figure. Gaps were introduced to optimize the alignment. This alignment was generated using the method of J. Hein, (Methods Enzymol. 1990 183 626-45) with PAM250 residue weight table. The boxed residues indicate amino acids which match the PDGF-Cs within two distance units.

The alignment shows that PDGF-C has the expected pattern of invariant cysteine residues, a hallmark of members of this family, with one exception. Between cysteine 3 and 4, normally spaced by 2 residues there is an insertion of three extra amino acids (NCA). This feature of the sequence in PDGF-C was highly unexpected.

Based on the amino acid sequence alignments in Figure 9, a phylogenetic tree was constructed and is shown in Figure 10. The data show that the PDGF-C homology domain is closely related to the PDGF/VEGF-homology domains of VEGF-C and VEGF-D.

As shown in Figure 11, the amino acid sequences from several CUB-containing proteins were aligned (SEQ ID NOs:18-24). The results show that the single CUB domain in human and mouse PDGF-C (SEQ ID NOs:18 and 19, respectively) displays a significant identify with the most closely related CUB domains. Sequences from human BMP-1, with 3 CUB domains (CUB1-

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3 (SEQ ID NOs:20-22)) and human neuropilin-1 with 2 CUB domains (CUB1-2) (SEQ ID NOs:23 and 24, respectively) are shown. Gaps were introduced to optimize the alignment. This alignment was generated using the method of J. Hein, (Methods Enzymol., 1990 183 626-45) with PAM250 residue weight table.

Figure 12 shows a Northern blot analysis of the expression of PDGF-C transcripts in several human tissues. The analysis shows that PDGF-C is encoded by a major transcript of approximately 3.8-3.9 kb, and a minor of 2.8 kb. The numbers to the right refer to the size of the mRNAs (in kb). The tissue expression of PDGF-C was determined by Northern blotting using a commercial Multiple Tissue Northern blot (MTN, Clontech). The blots were hybridized according to the instructions from the supplier using ExpressHyb solution at 68°C for one hour (high stringency conditions), and probed with a 353 bp hPDGF-C EST probe from the fetal lung cDNA library screening as described above. The blots were subsequently washed at 50°C in 2X SSC with 0.05% SDS for 30 minutes and at 50°C in 0.1X SSC with 0.1% SDS for an additional 40 minutes. The blots were then put on film and exposed at -70°C. The blots show that PDGF-C transcripts are most abundant in heart, liver, kidney, pancreas and ovary while lower levels of transcripts are present in most other tissues, including placenta, skeletal muscle and prostate. PDGF-C transcripts were below the level of detection in spleen, colon and peripheral blood leucocytes.

Figure 13 shows the regulation of PDGF-C mRNA expression by hypoxia. Size markers (in kb) are indicated to the left in the lower panel. The estimated sizes of PDGF-C mRNAs is indicated to the left in the upper panel (2.7 and 3.5 kbs, respectively). To explore whether PDGF-C is induced by hypoxia, cultured human skin fibroblasts were exposed to hypoxia for 0, 4, 8 and 24 hours. Poly(A)+ mRNA was isolated



5 from cells using oligo-dT cellulose affinity purification.  
Isolated mRNAs were electrophoresed through 12% agarose gels  
10 using 4 µg of mRNA per line. A Northern blot was made and  
hybridized with a probe for PDGF-C. The sizes of the two  
5 bands were determined by hybridizing the same filter with a  
mixture of hVEGF, hVEGF-B and hVEGF-C probes (Enholm et al.  
15 Oncogene, 1997 14 2475-2483), and interpolating on the basis  
of the known sizes of these mRNAs. The results shown in Figure  
13 indicate that PDGF-C is not regulated by hypoxia in human  
10 skin fibroblasts.

20 Figure 14 shows the expression of PDGF-C mRNA in human  
tumor cells lines. To explore whether PDGF-C was expressed  
in human tumor cell lines, poly(A)+ mRNA was isolated from  
several known tumor cell lines, the mRNAs were electrophoresed  
25 through a 12% agarose gel and analyzed by Northern blotting  
and hybridization with the PDGF-C probe. The results shown in  
Figure 14 demonstrate that PDGF-C mRNA is expressed in several  
types of human tumor cell lines such as JEG3 (a human  
30 choriocarcinoma, ATCC #HTB-36), G401 (a Wilms tumor, ATCC  
#CRL-1441), DAMI (a megakaryoblastic leukemia), A549 (a human  
lung carcinoma, ATCC #CCL-185) and HEL (a human  
35 erythroleukemia, ATCC #TID-180). It is contemplated that  
further growth of these PDGF-C expressing tumors can be  
inhibited by inhibiting PDGF-C. As well as using PDGF-C  
expression as a means of identifying specific types of tumors.  
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40 Example 1: Generation of specific antipeptide antibodies to  
human PDGF-C

45 30 Two synthetic peptides were generated and then used to  
raise antibodies against human PDGF-C. The first synthetic  
peptide corresponds to residues 29-48 of the N-terminus of  
full length PDGF-C and includes an extra cysteine residue at  
the N- and C-terminus: CKFQFSSNKEQNGVQDPQHERC (SEQ ID NO:31).  
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The second synthetic peptide corresponds to residues 230-250 of the internal region of full length PDGF-C and includes an extra cysteine residue at the C-terminus: GRKSRVVDLNLLEEVRVLYSC (SEQ ID NO:32). The two peptides were each conjugated to the carrier protein keyhole limpet hemocyanin (KLH, Calbiochem) using N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) (Pharmacia Inc.) according to the instructions of the supplier. 200-300 micrograms of the conjugates in phosphate buffered saline (PBS) were separately emulsified in Freund's Complete Adjuvant and injected subcutaneously at multiple sites in rabbits. The rabbits were boosted subcutaneously at biweekly intervals with the same amount of the conjugates emulsified in Freund's Incomplete Adjuvant. Blood was drawn and collected from the rabbits. The sera were prepared using standard procedures known to those skilled in the art.

Example 2: Expression of full length human PDGF-C in mammalian cells

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The full length cDNA encoding human PDGF-C was cloned into the mammalian expression vector, pSG5 (Stratagene, La Jolla, CA) that has the SV40 promoter. COS-1 cells were transfected with this construct and in separate transfections, with a pSG5 vector without the cDNA insert for a control, using the DEAE-dextran procedure. Serum free medium was added to the transfected COS-1 cells 24 hours after the transfections and aliquots containing the secreted proteins were collected for a 24 hour period after the addition of the medium. These aliquots were subjected to precipitation using ice cold 10% trichloroacetic acid for 30 minutes, and the precipitates were washed with acetone. The precipitated proteins were dissolved in SDS loading buffer under reducing conditions and separated on a SDS-PAGE gel using standard

procedures. The separated proteins were electrotransferred onto Hybond filter and immunoblotted using a rabbit antiserum against the internal peptide of full length PDGF-C, the preparation of which is described above. Bound antibodies were detected using enhanced chemiluminescence (ECL, Amersham Inc.). Figure 15 shows the results of this immunoblot. The sample was only partially reduced and the monomer of the human PDGF-C migrated as a 55 kDa species (the lower band) and the dimer migrated as a 100 kDa species (upper band). This indicates that the protein is secreted intact and that no major proteolytic processing occurs during secretion of the molecule in mammalian cells.

Example 3: Expression of full length and truncated human PDGF-C in baculovirus infected Sf9 cells

The full length coding part of the human PDGF-C cDNA (970 bp) was amplified by PCR using Deep Vent DNA polymerase (Biolabs) using standard conditions and procedures. The full length PDGF-C was amplified for 30 cycles, where each cycle consisted of one minute denaturization at 94°C, one minute annealing at 56°C and two minutes extension at 72°C. The forward primer used was 5'CGGGATCCCGAATCCAACCTGAGTAG3' (SEQ ID NO:33). This primer includes a BamHI site (underlined) for in frame cloning. The reverse primer used was 5'GGAATTCCTAATGGTGATGGTGATGATGTTTGTCATCGTCATCTCCTCCTGTGCTCCTCT3' (SEQ ID NO:34). This primer includes an EcoRI site (underlined) and sequences coding for a C-terminal 6X His tag preceded by an enterokinase site. In addition, residues 230-345 of the PDGF/VEGF homology domain (PVHD) of human PDGF-C were amplified by PCR using Deep Vent DNA polymerase (Biolabs) using standard conditions and procedures. The residues 230-345 of the PVHD of PDGF-C were amplified for 25 cycles, where each cycle consisted of one minute denaturization at 94°C,

four minutes annealing at 56°C and four minutes extension at 72°C. The forward primer used was 5'CGGATCCCGGAAGAAATCCA GAGTGGTG3' (SEQ ID NO:35). This primer includes a BamHI site (underlined) for in frame cloning. The reverse primer used was 5'GGAATTCCTAATGGTGATGGTGATGTTGTTCATCGTCATCTCCTCCTGTG CTCCTCT-3' (SEQ ID NO:36). This primer includes an EcoRI site (underlined) and sequences coding for a C-terminal 6X His tag preceded by an enterokinase site. The PCR products were digested with BamHI and EcoRI and subsequently cloned into the baculovirus expression vector, pAcGP67A. Verification of the correct sequence of the PCR products cloned into the constructs was by nucleotide sequencing. The expression vectors were then co-transfected with BaculoGold linearized baculovirus DNA into Sf9 insect cells according to the manufactures protocol (Pharmingen). Recombined baculovirus were amplified several times before beginning large scale protein production and protein purification according to the manual (Pharmingen).

Sf9 cells, adapted to serum free medium, were infected with recombinant baculovirus at a multiplicity of infection of about 7. Media containing the recombinant proteins were harvested 4 days after infection and were incubated with Ni-NTA-Agarose beads (Qiagen). The beads were collected in a column and after extensive washing with 50 mM sodium phosphate buffer pH 8, containing 300 mM NaCl (the washing buffer), the bound proteins were eluted with increasing concentrations of imidazole (from 100 mM to 500 mM) in the washing buffer. The eluted proteins were analyzed by SDS-PAGE using 12.5% polyacrylamide gels under reducing and non-reducing conditions. For immunoblotting analyses, the proteins were electrotransferred onto Hybond filters for 45 minutes.

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Figures 16A-C show the isolation and partial characterization of full length human PDGF-C protein. In Figure 16A, the recombinant full length protein was visualized on the blot using anti-peptide antibodies against the N-terminal peptide (described above). In Figure 16B, the recombinant full length protein was visualized on the blot using anti-peptide antibodies against the internal peptide (described above). The separated proteins were visualized by staining with Coomassie Brilliant Blue (Figure 16C). The numbers at the bottom of Figures 16A-C refer to the concentration of imidazole used to elute the protein from the Ni-NTA column and are expressed in molarity (M). Figures 16A-C also show that the full length protein migrates as a 90 kDa species under non-reducing conditions and as a 55 kDa species under reducing conditions. This indicates that the full length protein was expressed as a disulfide-linked dimer.

Figures 17A-C show the analysis of the isolation and partial characterization of a truncated form of human PDGF-C containing the PDGF/VEGF homology domain only. In Figure 17A, the immunoblot analysis of fractions eluted from the Ni-agarose column demonstrates that the protein could be eluted at imidazole concentrations ranging between 100-500 mM. The eluted fractions were analyzed under non-reducing conditions, and the truncated human PDGF-C was visualized on the blot using anti-peptide antibodies against the internal peptide (described above). Figure 17B shows the Coomassie Brilliant Blue staining of the same fractions as in Figure 17A. This shows that the procedure generates highly purified material migrating as a 36 kDa species. Figure 17C shows the Coomassie Brilliant Blue staining of non-reduced (non-red.) and reduced (red.) truncated human PDGF-C protein. The data show that the protein is a

secreted dimer held together by disulfide bonds and that the monomer migrates as a 24 kDa species.

Example 4: Receptor binding properties of full length and truncated PDGF-C

To assess the interactions between full length and truncated PDGF-C and the VEGF receptors, full length and truncated PDGF-C were tested for their capacity to bind to soluble Ig-fusion proteins containing the extracellular domains of human VEGFR-1, VEGFR-2 and VEGFR-3 (Olofsson et al., Proc. Natl. Acad. Sci. USA, 1998 95 11709-11714). The fusion proteins, designated VEGFR-1-Ig, VEGFR-2-Ig and VEGFR-3-Ig, were transiently expressed in human 293 EBNA cells. All Ig fusion proteins were human VEGFRs. Cells were incubated for 24 hours after transfection, washed with Dulbecco's Modified Eagle Medium (DMEM) containing 0.2% bovine serum albumin and starved for 24 hours. The fusion proteins were then precipitated from the clarified conditioned medium using protein A-Sepharose beads (Pharmacia). The beads were combined with 100 microliters of 10X binding buffer (5% bovine serum albumin, 0.2% Tween 20 and 10 µg/ml heparin) and 900 microliter of conditioned medium from 293 cells that had been transfected with mammalian expression plasmids encoding full length or truncated PDGF-C or control vector, then metabolically labeled with <sup>35</sup>S-cysteine and methionine (Promix, Amersham) for 4 to 6 hours. After 2.5 hours, at room temperature, the Sepharose beads were washed 3 times with binding buffer at 4°C, once with phosphate buffered saline and boiled in SDS-PAGE buffer. Labeled proteins that were bound to the Ig-fusion proteins were analyzed by SDS-PAGE under reducing conditions. Radiolabeled proteins were detected using a phosphorimager analyzer. In all these analyses,

radiolabeled PDGF-C failed to show any interaction with any of the VEGF receptors.

Next, full length and truncated PDGF-C were tested for their capacity to bind to human PDGF receptors alpha and beta by analyzing their abilities to compete with PDGF-BB for binding to PDGF receptors. The binding experiments were performed on porcine aortic endothelial-1 (PAE-1) cells stably expressing the human PDGF receptors alpha and beta (Eriksson et al., EMBO J, 1992, 11, 543-550). Binding experiments were performed essentially as in Heldin et al. (EMBO J, 1988, 7 1387-1393). Different concentrations of human full-length and truncated PDGF-C, or human PDGF-BB were mixed with 5 ng/ml of  $^{125}\text{I}$ -PDGF-BB in binding buffer (PBS containing 1 mg/ml of bovine serum albumin). Aliquots were incubated with the receptor expressing PAE-1 cells plated in 24-well culture dishes on ice for 90 minutes. After three washes with binding buffer, cell-bound  $^{125}\text{I}$ -PDGF-BB was extracted by lysis of cells in 20 mM Tris-HCl, pH 7.5, 10% glycerol, 1% Triton X-100. The amount of cell bound radioactivity was determined in a gamma-counter. A standard curve for the binding of  $^{125}\text{I}$ -labeled PDGF BB homodimers to PAE-1 cells expressing PDGF alpha-receptor is shown in Figure 18. An increasing excess of the unlabeled protein added to the incubations competed efficiently with cell association of the radiolabeled tracer.

Figure 19-graphically shows that the truncated PDGF-C efficiently competed for binding to the PDGF alpha-receptor, while the full length protein did not. Both the full length and truncated proteins failed to compete for binding to the PDGF beta-receptor.

#### Example 5: PDGF alpha-receptor Phosphorylation

To test if PDGF-C causes increased phosphorylation of

the PDGF alpha-receptor, full length and truncated PDGF-C were tested for their capacity to bind to the PDGF alpha-receptor and stimulate increased phosphorylation. Serum-starved porcine aortic endothelial (PAE) cells stably expressing the human PDGF alpha-receptor were incubated on ice for 90 minutes with PBS supplemented with 1 mg/ml BSA and 10ng/ml of PDGF-AA, 100ng/ml of full length human PDGF-CC homodimers (flPDGF-CC), 100ng/ml of truncated PDGF-CC homodimers (cPDGF-CC), or a mixture of 10ng/ml of PDGF-AA and 100ng/ml of truncated PDGF-CC. Full length and truncated PDGF-CC homodimers were produced as described above. Sixty minutes after the addition of the polypeptides, the cells were lysed in lysis buffer (20 mM tris-HCl, pH 7.5, 0.5% Triton X-100, 0.5% deoxycholic acid, 10 mM EDTA, 1 mM orthovanadate, 1 mM PMSF 1% Trasylol). The PDGF alpha-receptors were immunoprecipitated from cleared lysates with rabbit antisera against the human PDGF alpha-receptor (Eriksson et al., EMBO J, 1992 11 543-550). The precipitated receptors were applied to a SDS-PAGE gel. After SDS gel electrophoresis, the precipitated receptors were transferred to nitrocellulose filters, and the filters were probed with anti-phosphotyrosine antibody PY-20, (Transduction Laboratories). The filters were then incubated with horseradish peroxidase-conjugated anti-mouse antibodies. Bound antibodies were detected using enhanced chemiluminescence (ECL, Amersham Inc). The filters were then stripped and reprobed with the PDGF alpha-receptor rabbit antisera, and the amount of receptors was determined by incubation with horseradish peroxidase-conjugated anti-rabbit antibodies. Bound antibodies were detected using enhanced chemiluminescence (ECL, Amersham Inc). The probing of the filters with PDGF alpha-receptor antibodies confirmed that equal amounts of the receptor were present



in all lanes. PDGF-AA is included in the experiment as a control. Figure 20 shows that truncated, but not full length PDGF-CC, efficiently induced PDGF alpha-receptor tyrosine phosphorylation. This indicates that truncated PDGF-CC is a potent PDGF alpha-receptor agonist.

Example 6: Mitogenicity of PDGF-C for Fibroblasts

Figure 21 shows the mitogenic activities of truncated and full length PDGF-CC on fibroblasts. The assay was performed essentially as described in Mori et al., J. Biol. Chem., 1991 266 21158-21164. Serum starved human foreskin fibroblasts were incubated for 24 hours with 1 ml of serum-free medium supplemented with 1 mg/ml BSA and 3ng/ml, 10ng/ml or 30ng/ml of full length PDGF-CC (flPDGF-CC), truncated PDGF-CC (cPDGF-CC) or PDGF-AA in the presence of 0.2  $\mu$ M [3H]thymidine. After trichloroacetic acid (TCA) precipitation, the incorporation of [3H]thymidine into DNA was determined using a beta-counter. The results show that truncated PDGF-CC, but not full length PDGF-CC, is a potent mitogen for fibroblasts. PDGF-AA is included in the experiment as a control.

PDGF-C does not bind to any of the known VEGF receptors. PDGF-C is the only VEGF family member, thus far, which can bind to and increase phosphorylation of the PDGF alpha-receptor. PDGF-C is also the only VEGF family member, thus far, to be a potent mitogen of fibroblasts. These characteristics indicate that the truncated form of PDGF-C may not be a VEGF family member, but instead a novel PDGF. Furthermore, the full length protein is likely to be a latent growth factor that needs to be activated by proteolytic processing to release the active PDGF/VEGF homology domain. A putative proteolytic site is the

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dibasic motif found in residues 231-234 in the full length protein, residues -R-K-S-R-. This site is structurally conserved in a comparison between mouse and human PDGF-Cs (Figure 7). Preferred proteases include, but are not limited to, Factor X and enterokinase. The N-terminal CUB domain may be expressed as an inhibitory domain which might be used to localize this latent growth factor in some extracellular compartment (for example the extracellular matrix) and which is removed by limited proteolysis when need, for example during embryonic development, tissue regeneration, tissue remodelling including bone remodelling, active angiogenesis, tumor progression, tumor invasion, metastasis formation and/or wound healing.

15 Example 7: PDGF Receptors Binding of Truncated PDGF-C

To assess the interactions between truncated PDGF-C and the PDGF alpha and beta receptors, truncated PDGF-C was tested for its capacity to bind to porcine aortic endothelial-1 (PAE-1) cells expressing PDGF alpha or beta receptors, respectively (Eriksson et al., EMBO J, 1992, 11 543-550). The binding experiments were performed essentially as described in Heldin et al. (EMBO J, 1988, 7 1387-1393). Five micrograms of truncated PDGF-C protein in ten microliters of sodium borate buffer was radiolabeled using the Bolton-Hunter reagent (Amersham) to a specific activity of  $4 \times 10^5$  cpm/ng. Different concentrations of radiolabeled truncated PDGF-C, with or without added unlabeled protein, in binding buffer (PBS containing 1 mg/ml of bovine serum albumin) was added to the receptor expressing PAE-1 cells plated in 24-well culture dishes on ice for 90 minutes. After three washes with binding buffer, cell-bound  $^{125}\text{I}$ -labeled PDGF-C was extracted by lysis of cells in 20 mM Tris-HCl, pH 7.5, 10% glycerol, 1%

5 Triton X-100. The amount of cell-bound radioactivity was  
determined in a gamma-counter. Non-specific binding was  
10 estimated by including a 100-fold molar excess of truncated  
PDGF-C in some experiments. All binding data represents  
5 the mean of triplicate analyses and the experimental  
variation in the experiment varied between 10-15%. As seen  
15 in Figure 22, truncated PDGF-C binds to cells expressing  
PDGF alpha receptors, but not to beta receptor expressing  
cells. The binding was specific as radiolabeled PDGF-C was  
20 quantitatively displaced by a 100-fold molar excess of  
unlabeled protein.

Example 8: Protease Effects on Full length PDGF-C

25 To demonstrate that full length PDGF-C can be  
15 activated by limited proteolysis to release the PDGF/VEGF  
homology domain from the CUB domain, the full length  
protein was digested with different proteases. For  
30 example, full length PDGF-C was digested with plasmin in 20  
mM Tris-HCl (pH 7.5) containing 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, and  
20 0.01% Tween 20 for 1.5 to 4.5 hours at 37°C using two to  
three units of plasmin (Sigma) per ml. The released domain  
35 essentially corresponded in size to the truncated PDGF-C  
species previously produced in insect cells.  
Plasmin-digested PDGF-C and undigested full length PDGF-C  
25 were applied to a SDS-PAGE gel under reducing conditions.  
After SDS-PAGE gel electrophoresis, the respective proteins  
40 were transferred to a nitrocellulose filter, and the filter  
was probed using a rabbit anti-peptide antiserum to residues  
230-250 in full length protein (residues GRKSRVVDLNLLEEVR  
45 LYSC (SEQ ID NO:37) located in just N-terminal to the  
PDGF/VEGF homology domain). Bound antibodies were detected  
using enhanced chemiluminescence (ECL, Amersham Inc).  
Figure 23 shows the immunoblot with a 55 kDa undigested

full length protein and the plasmin-generated 26-28 kDa species.

Example 9: PDGF alpha Receptors Binding of Plasmin-digested PDGF-C

To assess the interactions between plasmin-digested PDGF-C and the PDGF alpha receptors, plasmin-digested PDGF-C was tested for its capacity to bind to porcine aortic endothelial-1 (PAE-1) cells expressing PDGF alpha receptors (Eriksson et al., EMBO J, 1992, 11 543-550). The receptor binding analyses were performed essentially as in Example 7 using 30 ng/ml of <sup>125</sup>I-labeled truncated PDGF-C as the tracer. As seen in Figure 24, increasing concentrations of plasmin-digested PDGF-C efficiently competed for binding to the PDGF alpha receptors. In contrast, undigested full length PDGF-C failed to compete for receptor binding. These data indicate that full length PDGF-C is a latent growth factor unable to interact with PDGF alpha receptors and that limited proteolysis, which releases the C-terminal PDGF/VEGF homology domain, is necessary to generate an active PDGF alpha receptor ligand/agonist.

Example 10: Cloning and expression of the Human PDGF-C CUB domain

A human PDGF-C 430 bp cDNA fragment encoding the CUB domain (amino acid residues 23 - 159 in full length PDGF-C) was amplified by PCR using Deep Vent DNA polymerase (Biolabs) using standard conditions and procedures. The forward primer used was 5'cgggatcccgaatccaacctgagtag3' (SEQ ID NO:38). This primer includes a BamHI site (underlined) for in clone frame cloning. The reverse primer used was 5'ccggaattcctaattggtgatggtgatggtgtttgtcatcgctcgacaatgttgta

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gtg3' (SEQ ID NO:39). This primer includes an EcoRI site (underlined) and sequences coding for a C-terminal 6x His tag preceded by an enterokinase site. The amplified PCR fragment was subsequently cloned into a pACgp67A transfer vector. Verification of the correct sequence of the expression construct, CUB-pACgp67A, was by automatic nucleotide sequencing. The expression vectors were then co-transfected with BaculoGold linearized baculovirus DNA into Sf9 insect cells according to the manufacture's protocol (Pharmingen). Recombined baculovirus were amplified several times before beginning large scale protein production and protein purification according to the manual (Pharmingen).

Sf9 cells, adapted to serum free medium, were infected with recombinant baculovirus at a multiplicity of infection of about 7. Media containing the recombinant proteins were harvested 72 hours after infection and were incubated with Ni-NTA-Agarose beads(Qiagen) overnight at 4°C. The beads were collected in a column and after extensive washing with 50 mM sodium phosphate buffer pH 8, containing 300 mM NaCl (the washing buffer), the bound proteins were eluted with increasing concentrations of imidazole (from 100 mM to 400 mM) in the washing buffer. The eluted proteins were analyzed by SDS-PAGE using a polyacrylamide gel under reducing and non-reducing conditions.

Figure 25 shows the results from Coomassie blue staining of the gel. The human PDGF-C CUB domain is a disulfide-linked homodimer with a molecular weight of about 55 KD under non-reducing conditions, while two monomers of about 25 and 30 KD respectively are present under reducing conditions. The heterogeneity is probably due to heterogenous glycosylation of the two putative N-linked glycosylation sites present in the CUB domain at amino acid

positions 25 and 55. A protein marker lane is shown to the left in the figure.

Example 11: Localization of PDGF-C transcripts in developing mouse embryos

To gain insight into the biological function of PDGF-C, PDGF-C expression in mouse embryos was localized by non-radioactive *in situ* hybridization in tissue sections from the head (Figures 26A-26S) and urogenital tract (Figures 26T-26V) regions. The non-radioactive *in situ* hybridization employed protocols and PDGF-A and PDGFR-alpha probes are described in Boström et al., Cell, 1996 85 863-873, which is hereby incorporated by reference. The PDGF-C probe was derived from a mouse PDGF-C cDNA. The hybridization patterns shown in Figures 26A-26V are for embryos aged E16.5, but analogous patterns are seen at E14.5, E15.5 and E17.5. Sense probes were used as controls and gave no consistent pattern of hybridization to the sections.

Figure 26A shows the frontal section through the mouth cavity at the level of the tooth anlagen (t). The arrows point to sites of PDGF-C expression in the oral ectoderm. Also shown is the tongue (to). Figures 26B-26D show PDGF-C expression in epithelial cells of the developing tooth canal. Individual cells are strongly labeled in this area (arrow in Figure 26D), as well as in the developing palate ectoderm (right arrow in Figure 26C). Figure 26E shows the frontal section through the eye, where PDGF-C expression is seen in the hair follicles (double arrow) and in the developing eyelid. Also shown is the retina (r). In Figures 26F and 26G, the PDGF-C expression is found in the outer root sheath of the developing hair follicle

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epithelium. In Figure 26H, PDGF-C expression is shown in the developing eyelid. There is an occurrence of individual strongly PDGF-C positive cells in the developing opening. Also shown is the lens (l). In Figure 26I, PDGF-C expression in the developing lacrimal gland is shown by the arrow. In Figure 26J, PDGF-C expression in the developing external ear is shown. Expression is seen in the external auditory meatus (left arrow) and in the epidermal cleft separating the prospective auricle (e). Figures 26K and 26L show PDGF-C expression in the cochlea. Expression is seen in the semi-circular canals (arrows in 26K). There is a polarized distribution of PDGF-C mRNA in epithelial cells adjacent to the developing hair cells (arrow in 26L). Figures 26M and 26N show PDGF-C expression in the oral cavity. Horizontal sections show expression in buccal epithelium (arrows in 26M) and in the forming cleft between the lower lip buccal and the gingival epithelium (arrows in 26N). Also shown is the tooth anlagen (t) and the tongue (to). Figures 26O and 26P show PDGF-C expression in the developing nostrils, shown on horizontal sections. PDGF-C expression appears strongest before stratification of the epithelium and the formation of the canal proper (arrows in 26O and 26P). Also shown is the developing nostrils (n). Figures 26Q-26S show PDGF-C expression in developing salivary glands and ducts. Figure 26Q is the sublingual gland. Figures 26R and 26S show the maxillary glands, the salivary gland (sg) and the salivary duct (sd). Figures 26T-26V show the expression of PDGF-C in the urogenital tract. Figure 26T shows the expression of PDGF-C in the developing kidney metanephric mesoderm. Figure 26U shows the expression of PDGF-C in the urethra (ua) and in epithelium surrounding the developing penis.

Figure 26V shows the PDGF-C expression in the developing ureter (u).

Example 12: PDGF-C, PDGF-A and PDGFR-alpha Expression in the Developing Kidney

One of the strongest sites of PDGF-C expression is the developing kidney and so expression of PDGF-C, PDGF-A and PDGFR-alpha was looked at in the developing kidney.

Figures 27A-27F show the results of non-radioactive *in situ* hybridization demonstrating the expression (blue staining in unstained background visualized using DIC optics) of mRNA for PDGF-C (Figures 27A and 27B), PDGF-A (Figures 27C and 27D) and PDGFR-alpha (Figures 27E and 27F) in E16.5 kidneys. The white hatched line in Figures 27B, 27D and 27F outlines the cortex border. The bar in Figures 27A, 27C and 27E represents 250  $\mu$ m, and in Figures 27B, 27D and 27F represents 50  $\mu$ m.

PDGF-C expression is seen in the metanephric mesenchyme (mm in Figure 27A), and appears to be upregulated in the condensed mesenchyme (arrows in Figure 27B) undergoing epithelial conversion as a prelude to tubular development, which is situated on each side of the ureter bud (ub). PDGF-C expression remains at lower levels in the early nephronal epithelial aggregates (arrowheads in B), but is absent from mature glomeruli (gl) and tubular structures.

PDGF-A expression is not seen in these early aggregates, but is strong in later stages of tubular development (Figures 24C and 24D). PDGF-A is expressed in early nephronal epithelial aggregates (arrowheads in Figure 27D), but once the nephron is developed further, PDGF-A expression becomes restricted to the developing Henle's loop (arrow in Figure 27D). The strongest expression is



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seen in the Henle's loops in the developing marrow (arrows in Figure 27C). The branching ureter (u) and the ureter bud (ub) is negative for PDGF-A.

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Thus, the PDGF-C and PDGF-A expression patterns in the developing nephron are spatially and temporally distinct. PDGF-C is expressed in the earliest stages (mesenchymal aggregates) and PDGF-A in the latest stages (Henle's loop formation) of nephron development.

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PDGFR-alpha is expressed throughout the mesenchyme of the developing kidney (Figures 27E and 27F) and may hence be targeted by both PDGF-C and PDGF-A. PDGF-B expression is also seen in the developing kidney, but occurs only in vascular endothelial cells. PDGFR-beta expression takes place in perivascular mesenchyme, and its activation by PDGF-B is critical for mesangial cell recruitment into glomeruli.

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These results demonstrate that PDGF-C expression occurs in close spatial relationship to sites of PDGFR-alpha expression, and are distinct from the expression sites of PDGF-A or PDGF-B. This indicates that PDGF-C may act through PDGFR-alpha in vivo, and may have functions that are not shared with PDGF-A and PDGF-B.

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Since the unique expression pattern of PDGF-C in the developing kidney indicates a function as a PDGFR-alpha agonist separate from that of PDGF-A or -B, a comparison was made to the histology of embryonic day 16.5 kidneys from PDGFR-alpha knockout mice (Figures 28B and 28F) with kidneys from wildtype (Figures 28A and 28C), PDGF-A knockout (Figure 28D) and PDGF-A/PDGF-B double knockout (Figure 28E) mice. The bar in Figures 28A and 28B represents 250  $\mu$ m, and in Figures 28C-28F represents 50  $\mu$ m.

Heterozygote mutants of PDGF-A, PDGF-B and PDGFR-alpha (Boström et al., Cell, 1996 85 863-873; Levéen et al.,

Genes Dev., 1994 8 1875-1887; Soriano et al., Development, 1997 124 2691-70) were bred as C57Bl6/129sv hybrids and intercrossed to produce homozygous mutant embryos. PDGF-A/PDGF-B heterozygote mutants were crossed to generate double PDGF-A/PDGF-B knockout embryos. Due to a high degree of lethality of PDGF-A  $-/-$  embryos before E10 (Boström et al., Cell, 1996 85 863-873), the proportion of double knockout E16.5 embryos obtained in such crosses were less than 1/40. The histology of kidney phenotypes was verified on at least two embryos of each genotype, except the PDGF-A/PDGF-B double knockout for which only a single embryo was obtained.

It is interesting that there is lack of interstitial mesenchyme in the cortex of PDGFR-alpha  $-/-$  kidney (arrows in Figure 28A and asterisk in Figure 28F) and the presence of interstitial mesenchyme in all other genotypes (asterisks in Figure 28C-E). The branching ureter (u) and the metanephric mesenchyme (mm) and its epithelial derivatives appear normal in all mutants. The abnormal glomerulus in the PDGF-A/PDGF-B double knockout reflect failure of mesangial cell recruitment into the glomerular tuft due to the absence of PDGF-B.

These results indicate that PDGFR-alpha knockouts have a kidney phenotype, which is not seen in PDGF-A or PDGF-A/PDGF-B knockouts, hence potentially reflecting loss of signaling by PDGF-C. The phenotype consists of the marked loss of interstitial mesenchyme in the developing kidney cortex. The cells lost in PDGFR-alpha  $-/-$  kidneys are thus normally PDGFR-alpha positive cells adjacent to the site of expression of PDGF-C.

#### BIOASSAYS TO DETERMINE THE FUNCTION OF PDGF-C

Assays are conducted to evaluate whether PDGF-C has

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similar activities to PDGF-A, PDGF-B, VEGF, VEGF-B, VEGF-C and/or VEGF-D in relation to growth and/or motility of connective tissue cells, fibroblasts, myofibroblasts and glial cells; to endothelial cell function; to angiogenesis; and to wound healing. Further assays may also be performed, depending on the results of receptor binding distribution studies.

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#### 10 I. Mitogenicity of PDGF-C for Endothelial Cells

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To test the mitogenic capacity of PDGF-C for endothelial cells, the PDGF-C polypeptide is introduced into cell culture medium containing 5% serum and applied to bovine aortic endothelial cells (BAEs) propagated in medium containing 10% serum. The BAEs are previously seeded in 24-well dishes at a density of 10,000 cells per well the day before addition of the PDGF-C. Three days after addition of this polypeptide the cells were dissociated with trypsin and counted. Purified VEGF is included in the experiment as positive control.

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#### II. Assays of Endothelial Cell Function

##### a) Endothelial cell proliferation

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Endothelial cell growth assays are performed by methods well known in the art, e.g. those of Ferrara & Henzel, Nature, 1989 380 439-443, Gospodarowicz et al., Proc. Natl. Acad. Sci. USA, 1989 86 7311-7315, and/or Claffey et al., Biochem. Biophys. Acta, 1995 1246 1-9.

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##### 30 b) Cell adhesion assay

The effect of PDGF-C on adhesion of polymorphonuclear granulocytes to endothelial cells is tested.

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c) *Chemotaxis*

The standard Boyden chamber chemotaxis assay is used to test the effect of PDGF-C on chemotaxis.

d) *Plasminogen activator assay*

Endothelial cells are tested for the effect of PDGF-C on plasminogen activator and plasminogen activator inhibitor production, using the method of Pepper et al., Biochem. Biophys. Res. Commun., 1991 181 902-906.

e) *Endothelial cell Migration assay*

The ability of PDGF-C to stimulate endothelial cells to migrate and form tubes is assayed as described in Montesano et al., Proc. Natl. Acad. Sci. USA, 1986 83 7297-7301. Alternatively, the three-dimensional collagen gel assay described in Joukov et al., EMBO J., 1996 15 290-298 or a gelatinized membrane in a modified Boyden chamber (Glaser et al., Nature, 1980 288 483-484) may be used.

III. *Angiogenesis Assay*

The ability of PDGF-C to induce an angiogenic response in chick chorioallantoic membrane is tested as described in Leung et al., Science, 1989 246 1306-1309. Alternatively the rat cornea assay of Rastinejad et al., Cell, 1989 56 345-355 may be used; this is an accepted method for assay of *in vivo* angiogenesis, and the results are readily transferrable to other *in vivo* systems.

IV. *Wound Healing*

The ability of PDGF-C to stimulate wound healing is tested in the most clinically relevant model available, as

described in Schilling et al., Surgery, 1959 46 702-710 and  
utilized by Hunt et al., Surgery, 1967 114 302-307.

#### V. The Haemopoietic System

A variety of *in vitro* and *in vivo* assays using  
specific cell populations of the haemopoietic system are  
known in the art, and are outlined below. In particular a  
variety of *in vitro* murine stem cell assays using  
fluorescence-activated cell sorter to purified cells are  
particularly convenient:

##### a) Repopulating Stem Cells

These are cells capable of repopulating the bone  
marrow of lethally irradiated mice, and have the Lin<sup>-</sup>, Rh<sup>h1</sup>,  
Ly-6A/E<sup>+</sup>, c-kit<sup>+</sup> phenotype. PDGF-C is tested on these cells  
either alone, or by co-incubation with other factors,  
followed by measurement of cellular proliferation by <sup>3</sup>H-  
thymidine incorporation.

##### b) Late Stage Stem Cells

These are cells that have comparatively little bone  
marrow repopulating ability, but can generate D13 CFU-S.  
These cells have the Lin<sup>-</sup>, Rh<sup>h1</sup>, Ly-6A/E<sup>+</sup>, c-kit<sup>+</sup> phenotype.  
PDGF-C is incubated with these cells for a period of time,  
injected into lethally irradiated recipients, and the  
number of D13 spleen colonies enumerated.

##### c) Progenitor-Enriched Cells

These are cells that respond *in vitro* to single growth  
factors and have the Lin<sup>-</sup>, Rh<sup>h1</sup>, Ly-6A/E<sup>+</sup>, c-kit<sup>+</sup> phenotype.  
This assay will show if PDGF-C can act directly on  
haemopoietic progenitor cells. PDGF-C is incubated with

these cells in agar cultures, and the number of colonies present after 7-14 days is counted.

#### VI. Atherosclerosis

Smooth muscle cells play a crucial role in the development or initiation of atherosclerosis, requiring a change of their phenotype from a contractile to a synthetic state. Macrophages, endothelial cells, T lymphocytes and platelets all play a role in the development of atherosclerotic plaques by influencing the growth and phenotypic modulations of smooth muscle cell. An *in vitro* assay using a modified Rose chamber in which different cell types are seeded on to opposite cover slips measures the proliferative rate and phenotypic modulations of smooth muscle cells in a multicellular environment, and is used to assess the effect of PDGF-C on smooth muscle cells.

#### VII. Metastasis

The ability of PDGF-C to inhibit metastasis is assayed using the Lewis lung carcinoma model, for example using the method of Cao et al., J. Exp. Med., 1995 182 2069-2077.

#### VIII. Migration of Smooth Muscle Cells

The effects of the PDGF-C on the migration of smooth muscle cells and other cells types can be assayed using the method of Koyama et al., J. Biol. Chem., 1992 267 22806-22812.

#### IX. Chemotaxis

The effects of the PDGF-C on chemotaxis of fibroblast, monocytes, granulocytes and other cells can be assayed

5 using the method of Siegbahn et al., J. Clin. Invest., 1990  
10 85 916-920.

15 **X. PDGF-C in Other Cell Types**

5 The effects of PDGF-C on proliferation,  
differentiation and function of other cell types, such as  
15 liver cells, cardiac muscle and other cells, endocrine  
cells and osteoblasts can readily be assayed by methods  
known in the art, such as <sup>3</sup>H-thymidine uptake by *in vitro*  
10 cultures. Expression of PDGF-C in these and other tissues  
can be measured by techniques such as Northern blotting and  
20 hybridization or by *in situ* hybridization.

25 **XI. Construction of PDGF-C Variants and Analogues**

15 PDGF-C is a member of the PDGF family of growth  
factors which exhibits a high degree of homology to the  
other members of the PDGF family. PDGF-C contains eight  
30 conserved cysteine residues which are characteristic of  
this family of growth factors. These conserved cysteine  
20 residues form intra-chain disulfide bonds which produce the  
cysteine knot structure, and inter-chain disulfide bonds  
35 that form the protein dimers which are characteristic of  
members of the PDGF family of growth factors. PDGF-C  
25 interacts with a protein tyrosine kinase growth factor  
40 receptor.

In contrast to proteins where little or nothing is  
known about the protein structure and active sites needed  
for receptor binding and consequent activity, the design of  
45 30 active mutants of PDGF-C is greatly facilitated by the fact  
that a great deal is known about the active sites and  
important amino acids of the members of the PDGF family of  
growth factors.

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Published articles elucidating the structure/activity relationships of members of the PDGF family of growth factors include for PDGF: Oestman et al., J. Biol. Chem., 1991 266 10073-10077; Andersson et al., J. Biol. Chem., 1992 267 11260-1266; Oefner et al., EMBO J., 1992 11 3921-3926; Flemming et al., Molecular and Cell Biol., 1993 13 4066-4076 and Andersson et al., Growth Factors, 1995 12 159-164; and for VEGF: Kim et al., Growth Factors, 1992 7 53-64; Pötgens et al., J. Biol. Chem., 1994 269 32879-32885 and Claffey et al., Biochem. Biophys. Acta, 1995 1246 1-9. From these publications it is apparent that because of the eight conserved cysteine residues, the members of the PDGF family of growth factors exhibit a characteristic knotted folding structure and dimerization, which result in formation of three exposed loop regions at each end of the dimerized molecule, at which the active receptor binding sites can be expected to be located.

Based on this information, a person skilled in the biotechnology arts can design PDGF-C mutants with a very high probability of retaining PDGF-C activity by conserving the eight cysteine residues responsible for the knotted folding arrangement and for dimerization, and also by conserving, or making only conservative amino acid substitutions in the likely receptor sequences in the loop 1, loop 2 and loop 3 region of the protein structure.

The formation of desired mutations at specifically targeted sites in a protein structure is considered to be a standard technique in the arsenal of the protein chemist (Kunkel et al., Methods in Enzymol., 1987 154 367-382). Examples of such site-directed mutagenesis with VEGF can be found in Pötgens et al., J. Biol. Chem., 1994 269 32879-32885 and Claffey et al., Biochem. Biophys. Acta, 1995 1246



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1-9. Indeed, site-directed mutagenesis is so common that kits are commercially available to facilitate such procedures (e.g. Promega 1994-1995 Catalog., Pages 142-145).

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5           The connective tissue cell, fibroblast, myofibroblast and glial cell growth and/or motility activity, the endothelial cell proliferation activity, the angiogenesis activity and/or the wound healing activity of PDGF-C mutants can be readily confirmed by well established screening procedures. For example, a procedure analogous to the endothelial cell mitotic assay described by Claffey et al., (Biochem. Biophys. Acta., 1995 1246 1-9) can be used. Similarly the effects of PDGF-C on proliferation of other cell types, on cellular differentiation and on human metastasis can be tested using methods which are well known in the art.

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20           The foregoing description and examples have been set forth merely to illustrate the invention and are not intended to be limiting. Since modifications of the disclosed embodiments incorporating the spirit and substance of the invention may occur to persons skilled in the art, the invention should be construed broadly to include all variations falling within the scope of the appended claims and equivalents thereof.

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## Claims

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What is claimed is:

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1. An isolated nucleic acid molecule comprising a polynucleotide sequence having at least 85% identity with the sequence of Figure 1, 3 or 5 (SEQ ID NOs: 2, 4 and 6, respectively).

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2. An isolated nucleic acid molecule according to claim 1, wherein the sequence identity is at least 90%.

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3. An isolated nucleic acid molecule according to claim 1, wherein the sequence identity is at least 95%.

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4. An isolated nucleic acid molecule according to claim 1, wherein said nucleic acid is a cDNA.

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5. An isolated nucleic acid molecule according to claim 1, wherein said nucleic acid is a mammalian polynucleotide.

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6. An isolated nucleic acid molecule according to claim 5, wherein said nucleic acid is a murine polynucleotide.

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7. An isolated nucleic acid molecule according to claim 6, comprising the sequence shown in Figure 5 (SEQ ID NO:6).

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8. An isolated nucleic acid molecule according to claim 5, wherein said nucleic acid is a human polynucleotide.

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9. An isolated nucleic acid molecule according to claim 8, comprising the sequence shown in Figure 1 or Figure 3 (SEQ ID NOs: 2 and 4, respectively). }

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10. An isolated nucleic acid molecule which encodes a polypeptide molecule comprising an amino acid sequence having at least 85% identity with the amino acid sequence of Figure 2 (SEQ ID NO:3) or the amino acid sequence of Figure 4 (SEQ ID NO:5), or a fragment or analog thereof having the biological activity of PDGF-C.

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11. An isolated nucleic acid molecule according to claim 10, wherein the amino acid sequence identity is at least 90%.

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12. An isolated nucleic acid molecule according to claim 10, wherein the amino acid sequence identity is at least 95%.

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13. An isolated nucleic acid molecule which codes for a polypeptide which comprises the amino acid sequence  
PXCXXVXRCCGXXXCC (SEQ ID NO:1).

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14. A vector comprising a nucleic acid according to claim 1, which nucleic acid is operably linked with a promoter sequence.

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15. A vector according to claim 14, wherein said vector is a eukaryotic vector.

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16. A vector according to claim 14, wherein said vector is a prokaryotic vector.

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17. A vector according to claim 14, wherein said vector is a plasmid.

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18. A vector according to claim 14, wherein said vector is a baculovirus vector.

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19. A method of making a vector which expresses a polypeptide comprising an amino acid sequence having at least 85% identity with the amino acid sequence of Figure 2 (SEQ ID NO:3) or the amino acid sequence of Figure 6 (SEQ ID NO:7), or fragment or analog thereof having the biological activity of PDGF-C, said method comprising incorporating an isolated nucleic acid according to claim 1, claim 10 or claim 13, into said vector in operatively linked relation with a promoter.

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20. A host cell transformed or transfected with a vector according to claim 14.

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21. A host cell according to claim 20, wherein said host cell is a eukaryotic cell.

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22. A host cell according to claim 20, wherein said host cell is a COS cell.

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23. A host cell according to claim 20, wherein said host cell is a prokaryotic cell.

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24. A host cell according to claim 20, wherein said host cell is a 293EBNA cell.

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25. A host cell according to claim 20, wherein said host cell is an insect cell.

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26. A host cell transformed or transfected with a vector comprising a nucleic acid sequence according to claim 1, operatively linked to a promoter, such that said host cell expresses a polypeptide comprising an amino acid sequence having at least 85% identity with the amino acid sequence of Figure 2 (SEQ ID NO:3) or the amino acid sequence of Figure 6 (SEQ ID NO:7), or a fragment or analog thereof having the biological activity of PDGF-C.

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27. An isolated polypeptide comprising at least 85% identity with the amino acid sequence of Figure 2 (SEQ ID NO:3) or the amino acid sequence of Figure 6 (SEQ ID NO:7), or a fragment or analog thereof having the biological activity of PDGF-C.

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28. An isolated polypeptide according to claim 27, wherein said polypeptide is a murine polypeptide.

29. An isolated polypeptide according to claim 27, wherein said polypeptide is a human polypeptide.

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30. An isolated polypeptide according to claim 27, wherein said polypeptide has the ability to stimulate and/or enhance proliferation and/or differentiation and/or growth and/or motility of cells expressing a PDGF-C receptor.

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31. An isolated polypeptide produced by expression of a polynucleotide comprising the polynucleotide sequence having at least 85% identity with the Figure 1, 3 or 5 (SEQ ID NOs: 2, 4 or 6, respectively), or a polynucleotide which hybridizes under stringent conditions with at least one of the foregoing DNA sequences.

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32. An isolated polypeptide which comprises the characteristic sequence

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PXCXXVXRCGGXXXRCC (SEQ ID NO:1).}

33. An isolated polypeptide dimer comprising a polypeptide according to claim 27.

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34. An isolated polypeptide dimer according to claim 33, wherein said polypeptide dimer is a homodimer of said polypeptide.

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35. An isolated polypeptide dimer according to claim 33, wherein said polypeptide dimer is a heterodimer of said polypeptide and VEGF, VEGF-B, VEGF-C, VEGF-D, PDGF-A, PDGF-B or PlGF.

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36. An isolated polypeptide dimer according to claim 33, wherein said polypeptide dimer is a disulfide-linked dimer.

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37. A pharmaceutical composition comprising an effective cell proliferation promoting amount of a polypeptide according to claim 27, claim 31 or claim 32, and at least one further growth factor selected from the group consisting of VEGF, VEGF-B, VEGF-C, VEGF-D, PDGF-A, PDGF-B or PlGF.

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38. A pharmaceutical composition according to claim 37, further comprising heparin.

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39. A pharmaceutical composition comprising an effective cell proliferation promoting amount of an isolated polypeptide according to claim 27, claim 31 or

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claim 32, and at least one pharmaceutical carrier or  
10 diluent.

40. A pharmaceutical composition comprising a PDGF  
receptor stimulating amount of an isolated polypeptide  
according to claim 27, claim 31 or claim 32, and at least  
15 one pharmaceutical carrier or diluent.

41. A pharmaceutical composition comprising an  
effective connective tissue or wound healing stimulating  
amount of an isolated polypeptide according to claim 27,  
20 claim 31 or claim 32, and at least one pharmaceutical  
carrier or diluent.

42. A means for amplifying a polynucleotide according  
to claim 1 in a test sample, said means comprising at least  
one pair of primers complementary to a nucleic acid  
25 according to claim 1.

43. A means for amplifying a polynucleotide as  
according to claim 1 in a test sample, said means  
comprising a polymerase and at least one pair of primers  
35 complementary to a nucleic acid according to claim 1, for  
amplifying the polynucleotide by polymerase chain reaction  
in order to facilitate a sequence comparison of the  
polynucleotide with the nucleic acid according to claim 1.  
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44. An antibody specifically reactive with a  
polypeptide according to claim 27, claim 31 or claim 32.  
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45. An antibody according to claim 44, wherein said  
antibody is a polyclonal antibody.  
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46. An antibody according to claim 44, wherein said antibody is a monoclonal antibody or a F(ab')<sub>2</sub>, F(ab'), F(ab) fragment or chimeric antibody.

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47. An antibody according to claim 45 or 46, wherein said antibody is labeled with a detectable label.

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48. An antibody according to claim 47, wherein said detectable label is radioactive isotope.

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49. A method of making a polypeptide according to claim 27, claim 31 or claim 32, said method comprising the steps of:

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culturing a host cell transformed or transfected with a vector comprising a polynucleotide encoding said polypeptide operably associated with a promoter sequence such that the nucleic acid sequence encoding said polypeptide is expressed; and

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isolating said polypeptide from said host cell or from a growth medium in which said host cell is cultured.

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50. A method of stimulating growth of connective tissue or wound healing in a mammal, comprising administering to said mammal an effective amount of the polypeptide according to claim 27, claim 31 or 32.

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51. A method for producing an active truncated form of PDGF-C, comprising the steps of expressing an expression vector comprising a polynucleotide encoding a polypeptide as claimed in claim 69.

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52. A method for regulating receptor-binding specificity of PDGF-C, comprising the steps of expressing

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an expression vector comprising a polynucleotide encoding a  
polypeptide as claimed in claim 27, claim 31 or claim 32  
10 and supplying a proteolytic amount of at least one enzyme  
for processing the expressed polypeptide to generate the  
active truncated form of PDGF-C.

15 53. A method for selectively activating a polypeptide  
having a growth factor activity comprising the step  
expressing an expression vector comprising a polynucleotide  
encoding a polypeptide having a growth factor activity, a  
20 CUB domain and a proteolytic site between the polypeptide  
and the CUB domain, and supplying a proteolytic amount of  
at least one enzyme for processing the expressed  
polypeptide to generate the active polypeptide having a  
25 growth factor activity.

30 54. An isolated polypeptide according to claim 27,  
claim 31 or claim 32 which comprises a proteolytic site  
having the amino acid sequence RKSR or a structurally  
conserved amino acid sequence thereof.

35 55. An isolated nucleic acid molecule according to  
claim 10 which codes for a polypeptide which comprises a  
proteolytic site having the amino acid sequence RKSR or a  
structurally conserved amino acid sequence thereof.

40 56. An isolated heterodimer comprising an active  
monomer of VEGF, VEGF-B, VEGF-C, VEGF-D, PDGF-C, PDGF-A,  
PDGF-B or PlGF and an active monomer of PDGF-C linked to a  
45 CUB domain.

50 57. An isolated heterodimer comprising an active  
monomer of PDGF-C and an activated monomer of VEGF, VEGF-B,

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VEGF-C, VEGF-D, PDGF-C, PDGF-A, PDGF-B or PlGF linked to a  
10 CUB domain.

58. An isolated heterodimer according to claim 56,  
further comprising a proteolytic site between the active  
monomer and the CUB domain linkage.

15 59. An isolated heterodimer according to claim 57,  
further comprising a proteolytic site between the active  
monomer and the CUB domain linkage.

20 60. An isolated polynucleotide, comprising a  
polynucleotide sequence having at least 85% identity with  
the sequence of Figure 1, 3 or 5 (SEQ ID NOS: 2, 4 or 6,  
25 respectively), or a polynucleotide which hybridize under  
stringent conditions with at least one of said DNA  
sequences.

30 61. A method of promoting fibroblast mitogenesis in a  
mammal, comprising the step of administering to said mammal  
an effective fibroblast mitogenesis promoting amount of the  
polypeptide according to claim 27, claim 31 or claim 32.

35 62. A method of inducing PDGF alpha receptor  
activation, comprising the step of adding a PDGF alpha-  
receptor stimulating amount of the polypeptide according to  
40 claim 27, claim 31 or claim 32.

45 63. A method of inhibiting tumor growth of a tumor  
expressing PDGF-C in a mammal, comprising administering to  
said mammal a PDGF-C inhibiting amount of a PDGF-C  
antagonist.

5

10

64. A method of identifying specific types of human tumors, comprising the step of taking a sample of the tumor and testing for the expression of PDGF-C.

15

65. The method of claim 64, wherein the specific types of tumors are selected from the group selected from choriocarcinoma, Wilms tumor, megakaryoblastic leukemia, lung carcinoma and erythroleukemia.

20

66. A method for identifying an PDGF-C antagonist comprising:

admixing a substantially purified preparation of an activated truncated form of PDGF-C with a test agent; and

25

monitoring, by any suitable means, an inhibition in the biological activity of PDGF-C.

30

67. A method for identifying an PDGF-C antagonist comprising:

admixing a substantially purified preparation of an full-length PDGF-C with a test agent; and

35

monitoring, by any suitable means, an inhibition in the cleavage of the CUB domain from PDGF-C.

40

68. An isolated polypeptide according to claim 27, wherein the cells are selected from the group consisting of endothelial cells, connective tissue cells, myofibroblasts and glial cells.

45

69. A method of making a vector which expresses a polypeptide comprising an amino acid sequence having at least 85% identity with the amino acid residues 230 to 345 of Figure 2 (SEQ ID NO:3) or of Figure 6 (SEQ ID NO:7),

50

55

5  
10 said method comprising incorporating an isolated nucleic acid molecule encoding said amino acid residues into said vector in operatively linked relation with a promoter.

15 70. An antibody according to claim 46, wherein said monoclonal antibody is a humanized antibody.

20 71. A method for producing an activated truncated form of PDGF-C, comprising the steps of expressing an expression vector comprising a polynucleotide encoding a polypeptide as claimed in claim 27, claim 31 or claim 32 and supplying a proteolytic amount of at least one enzyme for processing the expressed polypeptide to generate the activated truncated form of PDGF-C.

25 72. A method of inhibiting tissue remodeling during invasion of tumor cells into a normal population of cells, comprising administering to said mammal a PDGF-C inhibiting amount of a PDGF-C antagonist.

30 73. A method of treating fibrotic conditions in a mammal in need a such treatment, comprising administering to said mammal a PDGF-C inhibiting amount of a PDGF-C antagonist.

35 74. A method of claim 73, wherein the fibrotic conditions are found in the lung, kidney or liver.

**Fig. 1**

ccccgccgtg agtgagctct cccccagtc agccaaatga gcctcttcgg gcttctcctg 60  
gtgacatctg ccctggccgg ccagagacga gggactcagg cggaatccaa cctgagtagt 120  
aaattccagt tttccagcaa caaggaacag aacggagtac aagatcctca gcatgagaga 180  
attattactg tgtctactaa tggaagtatt cacagcccaa ggtttcctca tacttatcca 240  
agaaatacgg tcttggtatg gagattagta gcagtagagg aaaatgtatg gatacaactt 300  
acgtttgatg aaagatttgg gcttgaagac ccagaagatg acatattgcaa gtatgatttt 360  
gtagaagttg aggaaccacg tgatggaact atattagggc gctggtgtgg ttctggtact 420  
gtaccaggaa aacagatttc taaaggaaat caaattagga taagatttgt atctgatgaa 480  
tattttcctt ctgaaccagg gttctgcac cactacaaca ttgtcatgcc acaattcaca 540  
gaagctgtga gtccttcagt gctacccctc tcagctttgc cactggacct gcttaataat 600  
gctataactg ccttagtagt cttggaagac cttattcgat atcttgaacc agagagatgg 660  
cagttggact tagaagatct atataggcca acttggcaac ttcttggcaa ggcttttgtt 720  
tttgaagaa aatccagagt ggtggatctg aaccttctaa cagaggaggt aagattatac 780  
agctgcacac ctcgtaactt ctcagtgtcc ataaggaag aactaaagag aaccgatacc 840  
atcttctggc caggttgtct cctggttaaa cgtgtgtgtg ggaactgtgc ctgttgtctc 900  
cacaattgca atgaatgtca atgtgtccca agcaaagtta ctaaaaata ccacgaggtc 960  
cttcagttga gaccaaagac cgggtgtcagg ggattgcaca aatcactcac cgacgtggcc 1020  
ctggagcacc atgaggagtg tgactgtgtg tgcagaggga gcacaggagg atagccgcat 1080  
caccaccagc agctcttgcc cagagctgtg cagtgcagtg gctgattcta ttagagaacg 1140  
tatgcgttat ctccatcctt aatctcagtt gtttgcttca aggaccttct atcttcagga 1200

## Fig. 1 cont.

tttacagtgc attctgaaag agsagacatc aaacagaattc aggaattgtg caacagctct 1260  
tttgagagga ggcctaaagg acaggagaaa aggtcttcaa tcgtggaaag aaaattaaat 1320  
gttgatttaa atagatcacc agctagtttc agagttacca tgtacgtatt ccactagctg 1380  
ggttctgtat ttcagttctt tcgatacggc ttagggtaat gtcagtacag gaaaaaaact 1440  
gtgcaagtga gcacctgatt ccgttgctt gcttaactct aaagctccat gtcctgggccc 1500  
taaaatcgta taaaatctgg atttttttt tttttttgc tcatattcac atatgtaac 1560  
cagaacattc tatgtactac aaacctgggt tttaaaaagg aactatgttg ctatgaatta 1620  
aactgtgtc rtgctgatag gacagactgg atttttcata tttcttatta aaatttctgc 1680  
catttagaag aagagaacta cattcatggt ttggaagaga taaacctgaa aagaagagt 1740  
gccttatctt cactttatcg ataagtcagt ttatttgtt cattgtgtac atttttatat 1800  
tctccttttg acattataac tgttggttt tctaactctg ttaaatatat ctatttttac 1860  
caaaggatt taatattctt ttttatgaca acttagatca actattttta gcttggttaa 1920  
tttttctaaa cacaattgtt atagccagag gaacaaagat ggatataaaa atattgttg 1980  
cctggacaaa aatacatgta tntccatccc ggaatgggtc tagagttgga ttaaacctgc 2040  
attttaaaaa acctgaattg ggaanggaan ttggttaagg ttgccaaanc tttttgaaa 2100  
ataattaa 2108

Fig. 2

Met Ser Leu Phe Gly Leu Leu Leu Val Thr Ser Ala Leu Ala Gly Gln  
 1 5 10 15  
 Arg Arg Gly Thr Gln Ala Glu Ser Asn Leu Ser Ser Lys Phe Gln Phe  
 20 25 30  
 Ser Ser Asn Lys Glu Gln Asn Gly Val Gln Asp Pro Gln His Glu Arg  
 35 40 45  
 Ile Ile Thr Val Ser Thr Asn Gly Ser Ile His Ser Pro Arg Phe Pro  
 50 55 60  
 His Thr Tyr Pro Arg Asn Thr Val Leu Val Trp Arg Leu Val Ala Val  
 65 70 75 80  
 Glu Glu Asn Val Trp Ile Gln Leu Thr Phe Asp Glu Arg Phe Gly Leu  
 85 90 95  
 Glu Asp Pro Glu Asp Asp Ile Cys Lys Tyr Asp Phe Val Glu Val Glu  
 100 105 110  
 Glu Pro Ser Asp Gly Thr Ile Leu Gly Arg Trp Cys Gly Ser Gly Thr  
 115 120 125  
 Val Pro Gly Lys Gln Ile Ser Lys Gly Asn Gln Ile Arg Ile Arg Phe  
 130 135 140  
 Val Ser Asp Glu Tyr Phe Pro Ser Glu Pro Gly Phe Cys Ile His Tyr  
 145 150 155 160  
 Asn Ile Val Met Pro Gln Phe Thr Glu Ala Val Ser Pro Ser Val Leu  
 165 170 175  
 Pro Pro Ser Ala Leu Pro Leu Asp Leu Leu Asn Asn Ala Ile Thr Ala  
 180 185 190  
 Phe Ser Thr Leu Glu Asp Leu Ile Arg Tyr Leu Glu Pro Glu Arg Trp  
 195 200 205  
 Gln Leu Asp Leu Glu Asp Leu Tyr Arg Pro Thr Trp Gln Leu Leu Gly  
 210 215 220  
 Lys Ala Phe Val Phe Gly Arg Lys Ser Arg Val Val Asp Leu Asn Leu  
 225 230 235 240  
 Leu Thr Glu Glu Val Arg Leu Tyr Ser Cys Thr Pro Arg Asn Phe Ser  
 245 250 255  
 Val Ser Ile Arg Glu Glu Leu Lys Arg Thr Asp Thr Ile Phe Trp Pro  
 260 265 270  
 Gly Cys Leu Leu Val Lys Arg Cys Gly Gly Asn Cys Ala Cys Cys Leu  
 275 280 285



## Fig. 2 cont.

His Asn Cys Asn Glu Cys Gln Cys Val Pro Ser Lys Val Thr Lys Lys  
290 295 300

Tyr His Glu Val Leu Gln Leu Arg Pro Lys Thr Gly Val Arg Gly Leu  
305 310 315 320

His Lys Ser Leu Thr Asp Val Ala Leu Glu His His Glu Glu Cys Asp  
325 330 335

Cys Val Cys Arg Gly Ser Thr Gly Gly  
340 345

Fig. 3

cgggtaaat ccagttttcc agcaacaagg aacagaacgg agtacaagat cctcagcatg 60  
agagaattat tactgtgtct actaatggaa gtattcacag cccaaggttt cctcactatt 120  
atccaagaaa tacgggtctg gtatggagat tagtagcagt agaggaaaat gtatggatag 180  
aaattacgtt tgatgaaaga ttggggcttg aagacccaga agatgacata tgcaagtatg 240  
atttttaga agttgaggaa cccagtgtat gaactatatt agggcgctgg tgtggttctg 300  
gtactgtacc aggaaaacag atttctaaag gaaatcaaata taggataaga tttgtatctg 360  
atgaatattt tccttctgaa ccagggttct gcateccata caacattgtc atgccacaat 420  
tcacagaagc tgtgagtcct tcagtgtac ccccttcagc ttgccactg gacctgtta 480  
ataatgctat aactgccttt agtaccttg aagaccttat tcgatatctt gaaccagaga 540  
gatggcagtt ggacttagaa gatctatata ggccaacttg gcaacttctt ggcaaggctt 600  
ttgttttttg aagaaaatcc agagtgggtg atctgaacct tctaacagag gaggtaat 660  
tatcacgctg cacacctcgt aacttctcag tgtccataag ggaadgaacta aagagaaccg 720  
ataccatttt ctggccaggt tgtctcctgg ttaaaccgtg tggggggaac tgtgcctgtt 780  
gtctccacaa ttgcaatgaa tgtcaatgtg tcccaagcaa agttactaaa aaataccacg 840  
aggtccttca gttgagacca aasaccggtg tcaggggatt gcacaaatca ctcaccgacg 900  
tggccctgga gcaccatgag gagtgtgact gtgtgtgcag agggagcaca ggaggatagc 960  
cgcatcacca ccagcagctc ttgccagag ctgtgcagt cagtggctga ttctattaga 1020  
gaacgtatgc gttatctcca tcttaattct cagtgtttg cttcaaggac ctttcatctt 1080  
caggatttac agtgcattct gaaagaggag acatcaaaca gaattaggag ttgtgcaaca 1140  
gctcttttga gaggaggcct aaaggacagg agaaaaggtc ttcaatcgtg gaaagaaaat 1200

## Fig. 3 cont.

taaaatgtgt attaaataga tcaccagcta gtttcagagt taccatgtac gtattccact 1260  
agctgggttc tgtatttcag ttctttcgat acggcttagg gtaatgtcag tacaggaaaa 1320  
aaactgtgca agtgagcacc tgattccgtt gccttgctta actctaaagc tccatgtcct 1380  
gggcctaaaa tcgtataaaa tctggatttt tttttttttt ttgctcata ttcacatatg 1440  
taaaccagaa cattctatgt actacaaacc tggtttttaa aaaggaacta tgttgctatg 1500  
aattaaactt gtgtcatgct gataggacag actgga 1536

Fig. 4

Gly Lys Phe Gln Phe Ser Ser Asn Lys Glu Gln Asn Gly Val Gln Asp  
 1 5 10 15  
 Pro Gln His Glu Arg Ile Ile Thr Val Ser Thr Asn Gly Ser Ile His  
 20 25 30  
 Ser Pro Arg Phe Pro His Thr Tyr Pro Arg Asn Thr Val Leu Val Trp  
 35 40 45  
 Arg Leu Val Ala Val Glu Glu Asn Val Trp Ile Gln Leu Thr Phe Asp  
 50 55 60  
 Glu Arg Phe Gly Leu Glu Asp Pro Glu Asp Asp Ile Cys Lys Tyr Asp  
 65 70 75 80  
 Phe Val Glu Val Glu Glu Pro Ser Asp Gly Thr Ile Leu Gly Arg Trp  
 85 90 95  
 Cys Gly Ser Gly Thr Val Pro Gly Lys Gln Ile Ser Lys Gly Asn Gln  
 100 105 110  
 Ile Arg Ile Arg Phe Val Ser Asp Glu Tyr Phe Pro Ser Glu Pro Gly  
 115 120 125  
 Phe Cys Ile His Tyr Asn Ile Val Met Pro Gln Phe Thr Glu Ala Val  
 130 135 140  
 Ser Pro Ser Val Leu Pro Pro Ser Ala Leu Pro Leu Asp Leu Leu Asn  
 145 150 155 160  
 Asn Ala Ile Thr Ala Phe Ser Thr Leu Glu Asp Leu Ile Arg Tyr Leu  
 165 170 175  
 Glu Pro Glu Arg Trp Gln Leu Asp Leu Glu Asp Leu Tyr Arg Pro Thr  
 180 185 190  
 Trp Gln Leu Leu Gly Lys Ala Phe Val Phe Gly Arg Lys Ser Arg Val  
 195 200 205  
 Val Asp Leu Asn Leu Leu Thr Glu Glu Val Arg Leu Tyr Ser Cys Thr  
 210 215 220  
 Pro Arg Asn Phe Ser Val Ser Ile Arg Glu Glu Leu Lys Arg Thr Asp  
 225 230 235 240  
 Thr Ile Phe Trp Pro Gly Cys Leu Leu Val Lys Arg Cys Gly Gly Asn  
 245 250 255  
 Cys Ala Cys Cys Leu His Asn Cys Asn Glu Cys Gln Cys Val Pro Ser  
 260 265 270  
 Lys Val Thr Lys Lys Tyr His Glu Val Leu Gln Leu Arg Pro Lys Thr  
 275 280 285

## Fig. 4 cont.

Gly	Val	Arg	Gly	Leu	His	Lys	Ser	Leu	Thr	Asp	Val	Ala	Leu	Glu	His
290						295					300				
His	Glu	Glu	Cys	Asp	Cys	Val	Cys	Arg	Gly	Ser	Thr	Gly	Gly		
305					310					315					

Fig. 5

cacctggaga cacagaagag ggcctctagga aazatttttg atgssgatta tgtggaaact 60  
accctgcgat tctctgctgc cagagccggc caggcgcttc caccgcagcg cagcctttcc 120  
ccgggctggg ctgagccttg gagtgcgcgc tccccagtg cccgcgcga gtgagccctc 180  
gccccagtca gccaaatgct cctcctcggc ctccctctgc tgacatctgc cctggccggc 240  
caaagaacgg ggactcgggc tgagtccaac ctgagcagca agttgcagct ctccagcgac 300  
aaggaacaga acggagtgc aatccccgg catgagagag ttgtcactat atctggtaat 360  
gggagcatcc acagcccgaa gtttcctcat acgtacccaa gaaatatggt gctgggtgtg 420  
agattagtgt cagtagatga aatgtgcgg atccagctga catttgatga gagatttggg 480  
ctggaagatc cagaagacga tatatgcaag tatgattttg tagaagtga ggagcccagt 540  
gatggaagtg ttttaggacg ctggtgtggt tctgggactg tgccaggaaa gcagacttct 600  
aaaggaaatc atatcaggat aagatttgta tctgatgagt attttccatc tgaaccggga 660  
ttctgcattc actacagtat tatcatgcca caagtcacag aaaccacgag tccttcgggtg 720  
ttgccccctt catctttgtc attggacctg ctcaacaatg ctgtgactgc cttcagtacc 780  
ttggaagagc tgattcggta cctagagcca gatcgatggc aggtggactt ggacagcctc 840  
tacaagccaa catggcagct tttgggcaag gctttcctgt atgggaaaaa aagcaaagt 900  
gtgaatctga atctcctcaa ggaagaggta aaactctaca gctgcacacc ccggaacttc 960  
tcagtgtcca tacgggaaga gctaaagagg acagatacca tattctggcc aggttgtctc 1020  
ctggtcaagc gctgtggagg aaattgtgcc tgtgtctcc ataattgcaa tgaatgtcag 1080  
tgtgtccac gtaaagtac aaaaagtac catgaggtcc ttcagttgag accaaaaact 1140  
ggagtcaagg gattgcataa gtcactcact gatgtggctc tggaacacca cgaggaaatg 1200

**Fig. 5 cont.**

gactgtgtgt gtagaggaaa cgcaggaggg taactgcagc ettcgtagca gcacacgtga 1260  
gcactggcat.tctgtgtacc cccacaagca accttcattc ccaccagcgt tgscgcgagg 1320  
gctctcagct gctgatgctg gctatggtaa agatcttact cgtctccaac caaattctca 1380  
gttgtttgc tcaatagcct tcccctgcag gacttcaagt gtcttctaaa agaccagagg 1440  
caccaanagg agtcaatcac aaagcactgc accg 1474

Fig. 6

Met Leu Leu Leu Gly Leu Leu Leu Leu Thr Ser Ala Leu Ala Gly Gln  
 1 5 10 15  
 Arg Thr Gly Thr Arg Ala Glu Ser Asn Leu Ser Ser Lys Leu Gln Leu  
 20 25 30  
 Ser Ser Asp Lys Glu Gln Asn Gly Val Gln Asp Pro Arg His Glu Arg  
 35 40 45  
 Val Val Thr Ile Ser Gly Asn Gly Ser Ile His Ser Pro Lys Phe Pro  
 50 55 60  
 His Thr Tyr Pro Arg Asn Met Val Leu Val Trp Arg Leu Val Ala Val  
 65 70 75 80  
 Asp Glu Asn Val Arg Ile Gln Leu Thr Phe Asp Glu Arg Phe Gly Leu  
 85 90 95  
 Glu Asp Pro Glu Asp Asp Ile Cys Lys Tyr Asp Phe Val Glu Val Glu  
 100 105 110  
 Glu Pro Ser Asp Gly Ser Val Leu Gly Arg Trp Cys Gly Ser Gly Thr  
 115 120 125  
 Val Pro Gly Lys Gln Thr Ser Lys Gly Asn His Ile Arg Ile Arg Phe  
 130 135 140  
 Val Ser Asp Glu Tyr Phe Pro Ser Glu Pro Gly Phe Cys Ile His Tyr  
 145 150 155 160  
 Ser Ile Ile Met Pro Gln Val Thr Glu Thr Thr Ser Pro Ser Val Leu  
 165 170 175  
 Pro Pro Ser Ser Leu Ser Leu Asp Leu Leu Asn Asn Ala Val Thr Ala  
 180 185 190  
 Phe Ser Thr Leu Glu Glu Leu Ile Arg Tyr Leu Glu Pro Asp Arg Trp  
 195 200 205  
 Gln Val Asp Leu Asp Ser Leu Tyr Lys Pro Thr Trp Gln Leu Leu Gly  
 210 215 220  
 Lys Ala Phe Leu Tyr Gly Lys Lys Ser Lys Val Val Asn Leu Asn Leu  
 225 230 235 240  
 Leu Lys Glu Glu Val Lys Leu Tyr Ser Cys Thr Pro Arg Asn Phe Ser  
 245 250 255  
 Val Ser Ile Arg Glu Glu Leu Lys Arg Thr Asp Thr Ile Phe Trp Pro  
 260 265 270  
 Gly Cys Leu Leu Val Lys Arg Cys Gly Gly Asn Cys Ala Cys Cys Leu  
 275 280 285



## Fig. 6 cont.

His Asn Cys Asn Glu Cys Gln Cys Val Pro Arg Lys Val Thr Lys Lys  
290 295 300

Tyr His Glu Val Leu Gln Leu Arg Pro Lys Thr Gly Val Lys Gly Leu  
305 310 315 320

His Lys Ser Leu Thr Asp Val Ala Leu Glu His His Glu Glu Cys Asp  
325 330 335

Cys Val Cys Arg Gly Asn Ala Gly Gly  
340 345

Fig. 7

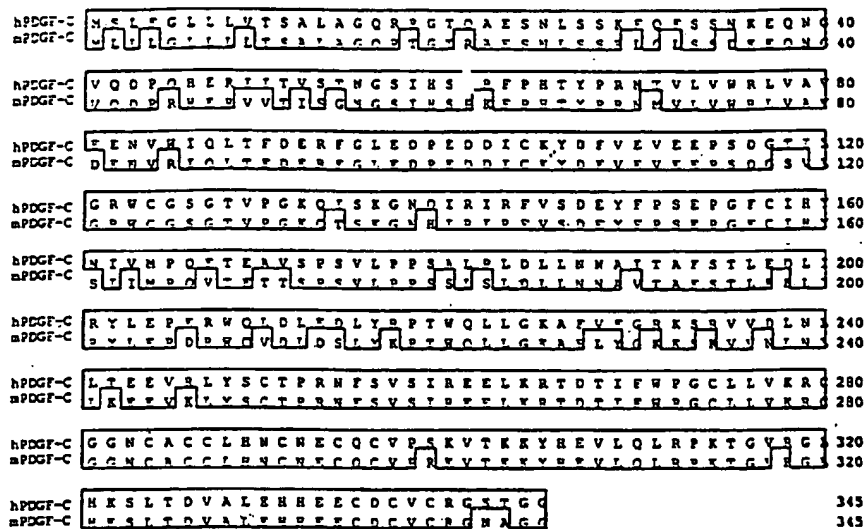


Fig. 8

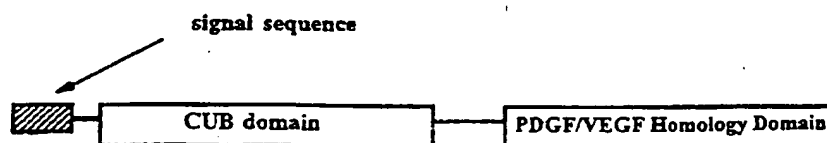


Fig. 9

VEGF 165	-----1
PlGF-2	-----1
VEGF-B167	-----1
Pox Cx1 VEGF	-----1
VEGF-C	MHLLGFFSVACSLLA AALLP GPREAPAAAA 30
VEGF-D	-----15
PlGF-A	-----1
PlGF-B	-----1
hPDGF-C PVDV	-----1
mPDGF-C PVDV	-----1
VEGF 165	-----1
PlGF-2	-----1
VEGF-B167	-----1
Pox Cx1 VEGF	-----1
VEGF-C	AFESGLDLSDAEPDAGEATAYAS KDLE EQL 60
VEGF-D	VYLVGGFRSEHGSPVKDFSPERSRSMLERS 45
PlGF-A	-----26
PlGF-B	MNRCWALFLSLCCYLRLVSAEGDPIPEELY 30
hPDGF-C PVDV	-----23
mPDGF-C PVDV	-----23
VEGF 165	-----10
PlGF-2	-----10
VEGF-B167	-----5
Pox Cx1 VEGF	-----1
VEGF-C	RSVSSVDELMTV DYPEYWKMYKCOL RKGGW 90
VEGF-D	EQOIRAAASSLEELLOTAHSEDWKLWRCRLK 75
PlGF-A	REVLERLARSQIHESI RDLQRLLEIDS VGSE 56
PlGF-B	EMLS DSHSIRSFDD LORLLHGD P-----GEE 55
hPDGF-C PVDV	NNA TAFSTLEDD IRYLEPERWQLDLEDLY 53
mPDGF-C PVDV	NNAVTA FSTLEET IRYLEPDRWQVDLDSLY 53
VEGF 165	SLA LLLYLHHA KWSQAAPMA EGGGQNHHEV 40
PlGF-2	LQL LAGLALPAVPPQOWALS AGNGSS EVEV 40
VEGF-B167	RRLLLAALLQL APAQAPVSQPDAPGHQRKV 35
Pox Cx1 VEGF	-----24
VEGF-C	QHNREQANLNS RTEETIEFAAAH YNTEI L 119
VEGF-D	LKSLASMDSRSAHRSTRFAATFYDTET L 104
PlGF-A	DSLDTSLRAHGVH--ATKHVPEKRPLPIRR 84
PlGF-B	DGAELDLNMTRSHSGGELES LARGRRSLGS 85
hPDGF-C PVDV	RPTWQLLGKAFVFG RKS R-----VVD L 75
mPDGF-C PVDV	KPTWQLLGKAFLYGKSK-----VVN L 75
VEGF 165	VKFM DVYQ RSYCHRIETLV DIFQ EYPDEIE 70
PlGF-2	VPFQ EVWGRSYCRALERLV D VSEYPSEVE 70
VEGF-B167	VSWIDVYTBATCQPREVVV PLTVEI MGVA 65
Pox Cx1 VEGF	-----54
VEGF-C	KGWS EVLKGSECKPRPIVVV VSETHPEL TS 149
VEGF-D	KSIDNEWKKTQC MPREV CIDVGKEE GVATN 134
PlGF-A	KVIDEEWORTOCSPRETCEVASELGKTTN 114
PlGF-B	KRSIEEAVPAVC KTRTVIY EIPRSQVDPTN 115
hPDGF-C PVDV	LTAEPAMIAEC KTRTEVE EISRR LIDRTN 104
mPDGF-C PVDV	NLLTEEVRLYSCTPRNFSVSI-REELKRTD 104
	NLLKEEVKLYSC TPRNFSVSI-REELKRTD 104

Fig. 9 cont.

VEGF 165	Y I F K - - P S C V F L M R C G G - - - C C N D E G L E C V 95
PLGF-2	H M E S - - P S C V S L L R C T G - - - C C G D E D L H C V 95
VEGF-B167	K Q L V - - P S C V T Q R C G G - - - C C P D D G L E C V 90
Pox Orl VEGF	Q R F N - - P P C V T L M R C G G - - - C C M D E S L E C V 79
VEGF-C	T F F K - - P P C V S V Y R C G G - - - C C M S E G L Q C M 174
VEGF-D	T F F K - - P P C V N V F R C G G - - - C C N E E G V M C M 159
PDGF-A	A N F L I W P P C V E V R C T G - - - C C M T S S V K C Q 141
PDGF-B	A N F L V W P P C V E V Q R C S G - - - C C N N R N V Q C R 142
hPDGF-C FVDV	T I F - - W P G C L L V R C G G N C A C C L H N C N E C Q 132
mPDGF-C FVDV	T I F - - W P G C L L V R C G G N C A C C L E N C N E C Q 132
VEGF 165	P T E E S N T M Q I M R I K - - - P H Q G Q - - - - H I 117
PLGF-2	P V E T A N V T M Q L L K I R - - - S G D R P - - - - S Y 117
VEGF-B167	P T G Q H Q V R M Q I L M I R Y - - - P S S Q L - - - - 111
Pox Orl VEGF	P T E E V N V S M E L L G A S G S C S N G M Q - - - - R L 104
VEGF-C	N T S T S Y L S K T L F E I T V - - - P L S O G - - - - P K 197
VEGF-D	N T S T S Y I S K O L F E I S V - - - P L T S V - - - - P E 182
PDGF-A	P S R V H H R S V K V A K V E Y V R K K P K L - - - - K E 166
PDGF-B	P T O V Q L R P V Q V R K T E I V R K K P I F - - - - K K 167
hPDGF-C FVDV	C V P - S K V T K K Y H E V L Q L R P K T G V R G L H K S L 161
mPDGF-C FVDV	C V P - R K V T K K Y H E V L Q L R P K T G V R G L H K S L 161
VEGF 165	G E M S F L Q H N R - C E C R P K K - - - - - D R 136
PLGF-2	V E L T F S Q H V R - C E C R P L R E - - - - K M K P E R R 142
VEGF-B167	G E M S L E E H S Q - C E C R P K K K - - - - D S A V K P 135
Pox Orl VEGF	S F V E H K K - - - C D C R P R F T - - - - T T P P 123
VEGF-C	P V T I S F A N H T S C R C M S K L D - - - V Y R Q V H S I 224
VEGF-D	L V P V K T A N H T G C K C L P T G P - - - - R H P Y S I 207
PDGF-A	V Q V R L E E H L E - C A C A T T S L N P D Y R E E D T G R 195
PDGF-B	A T V T L E D H L A - C K C E T V A A A R P V T R S P G G S 196
hPDGF-C FVDV	T D V A L E H H E E - C D C V C R G S T G G 182
mPDGF-C FVDV	T D V A L E H H E E - C D C V C R G N A G G 182
VEGF 165	A R Q E N P C G P C S S E R R K H L F V Q D P Q T C K C S C 166
PLGF-2	R P K G R G K R R R E N Q R P T D C H L C G D A V P R R 170
VEGF-B167	D S P R P L C R C T Q H H Q R P D P R T - - - - C R C R C 161
Pox Orl VEGF	T T T R P P R R R R 133
VEGF-C	I R R S L P A T - L P Q C Q A A N R T C P T N Y M W N N H I 253
VEGF-D	I R R S T O T P E E D E C P H S K K L C P I D M L W D N T K 237
PDGF-A	P R E S G K K R K R K R L K P T 211
PDGF-B	Q E Q R A K T E Q T R V T I R T V R V R R P P K G K H R K F 225
hPDGF-C FVDV	182
mPDGF-C FVDV	182
VEGF 165	K N T D S - R C K A R Q L E L N E R T C R C D K P R R 192
PLGF-2	170
VEGF-B167	R R R S F L R C Q G R G L E L N P D T C R C R K L R R 188
Pox Orl VEGF	133
VEGF-C	C R C L A Q E D F M F S S D A G D D S T D G F H D I C G P N 283
VEGF-D	C K C V L O D E - T P L P G T E D H S Y L O E P T L C G P H 266
PDGF-A	211
PDGF-B	K H T H D K T A L K E T L G A 241
hPDGF-C FVDV	182
mPDGF-C FVDV	182

Fig. 9 cont.

VEGF 165	192
PLGF-2	170
VEGF-B167	188
Pox Orf VEGF	133
VEGF-C	313
VEGF-D	273
PDGF-A	211
PDGF-B	241
hPDGF-C FVDV	182
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VEGF 165	192
PLGF-2	170
VEGF-B167	188
Pox Orf VEGF	133
VEGF-C	343
VEGF-D	297
PDGF-A	211
PDGF-B	241
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VEGF 165	192
PLGF-2	170
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PLGF-2	170
VEGF-B167	188
Pox Orf VEGF	133
VEGF-C	403
VEGF-D	338
PDGF-A	211
PDGF-B	241
hPDGF-C FVDV	182
mPDGF-C FVDV	182
VEGF 165	192
PLGF-2	170
VEGF-B167	188
Pox Orf VEGF	133
VEGF-C	419
VEGF-D	358
PDGF-A	211
PDGF-B	241
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Fig. 10

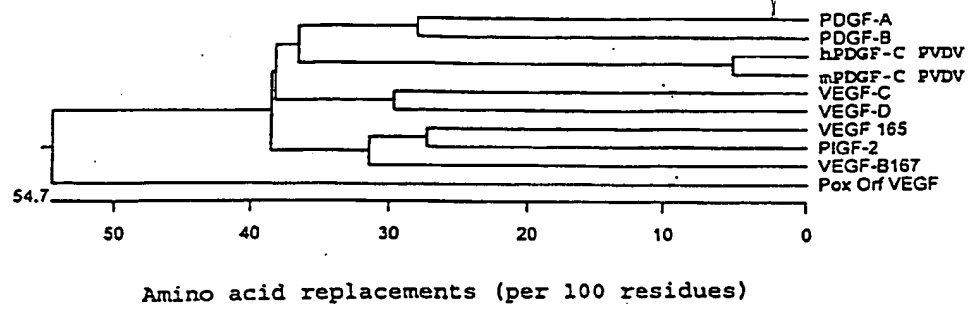


Fig. 11

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hP0G7-1 C0E2	CGGDVKKDYGMQSPHYDDYSPSKQCIWRZOVSKCF-HV	470
hP0G7-1 C0E3	CGGFLTKLNGSNTSPGWKEYFPKNCIMQYVAPTQY-RZ	620
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Neuropilin C0E2	CSQNYTTPSGVAKSGTGERKYNSELECTYIVFAPKHSE-A	175
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hP0G7-1 C0E2	CLTFQS-FEIRND-----SCATDYVEVRDCYWRKAPLR	506
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Fig. 12

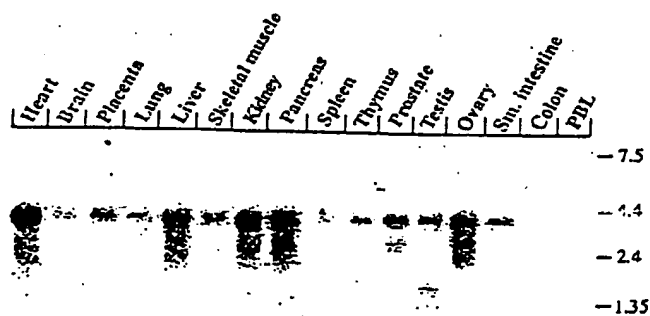


Fig. 13

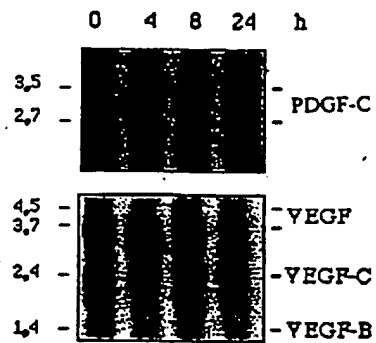


Fig. 14

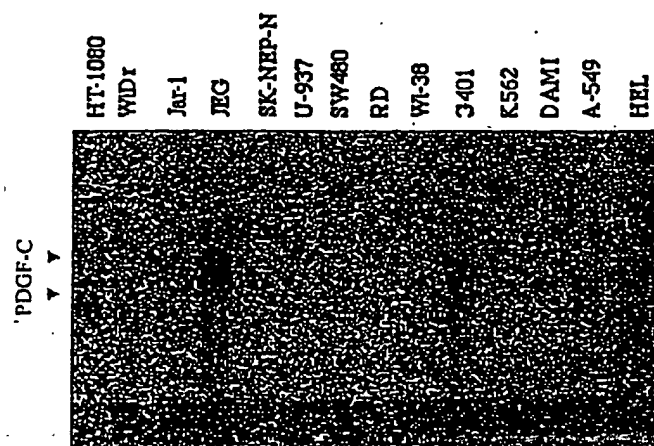




Fig. 15

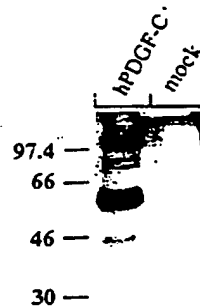


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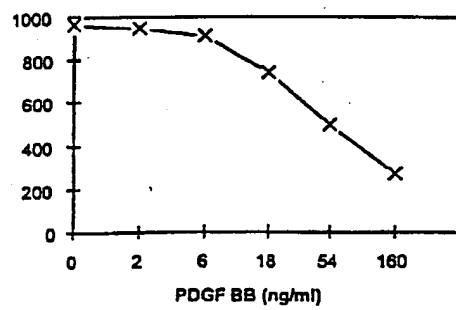


Fig. 19

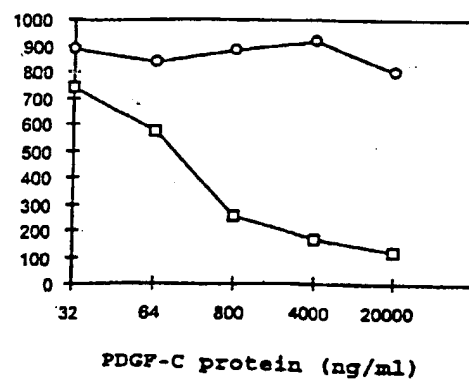


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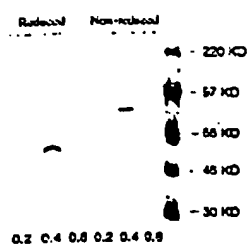


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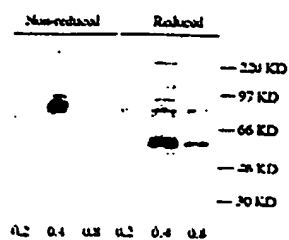


Fig. 16C

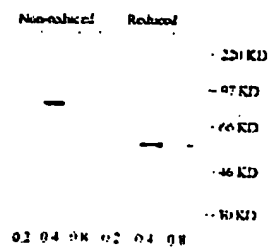


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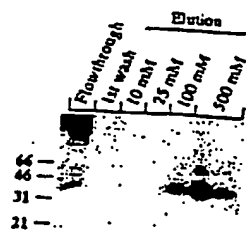


Fig. 17B

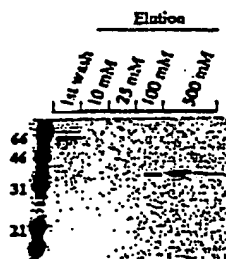


Fig. 17C



Fig. 20

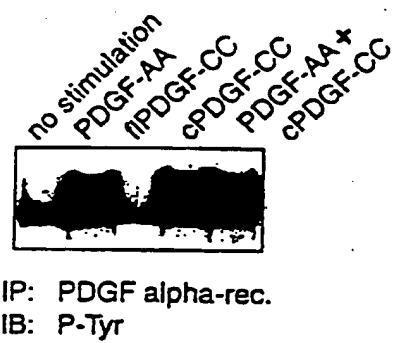


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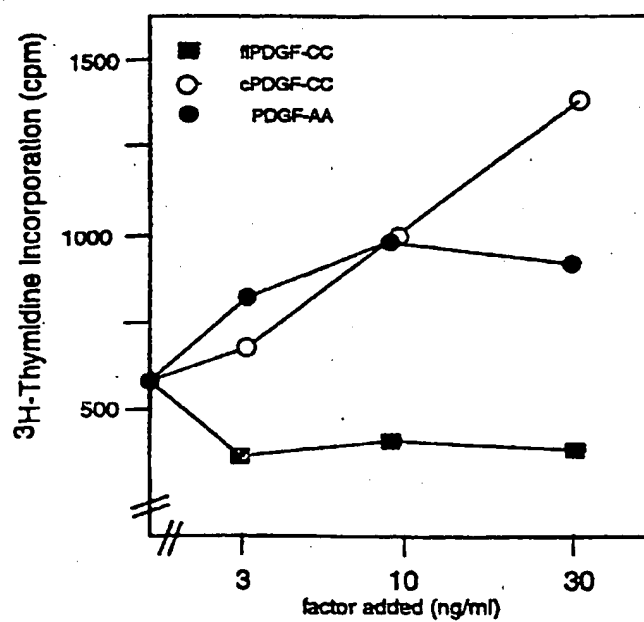


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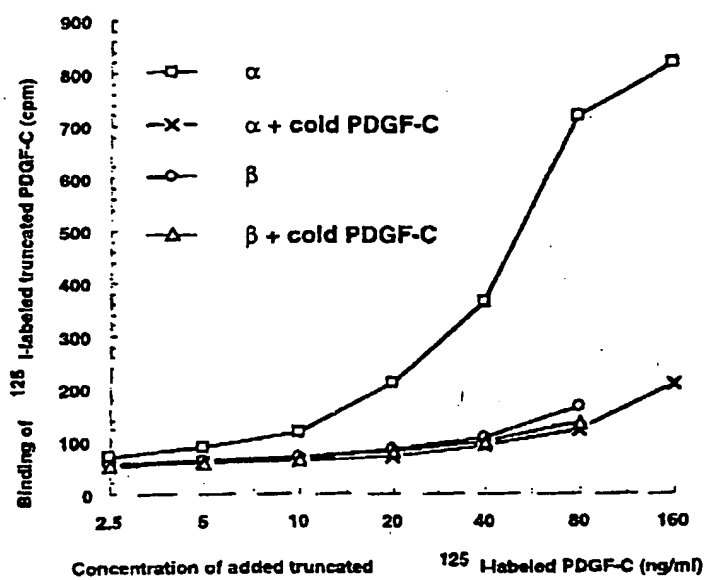


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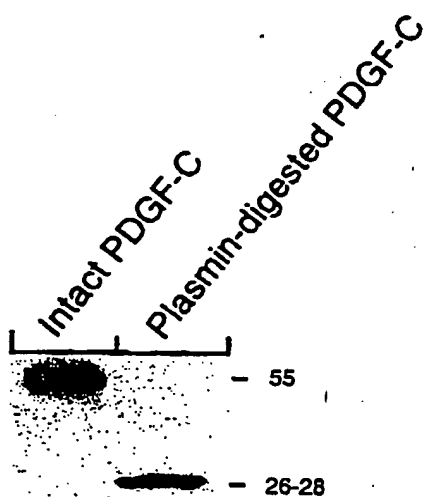


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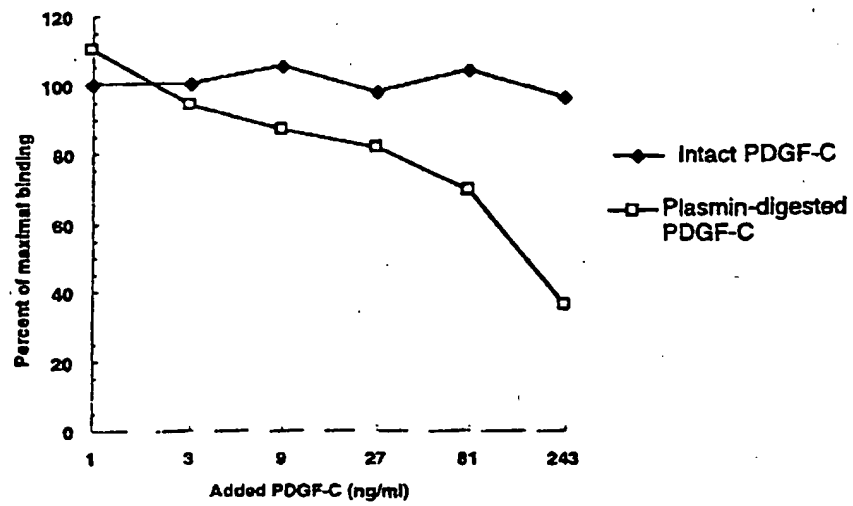


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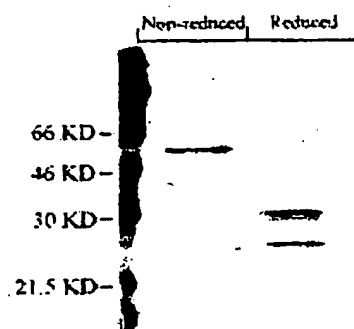




Fig. 26A

Fig. 26B

Fig. 26C

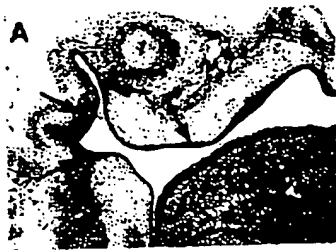


Fig. 26D

Fig. 26E

Fig. 26F

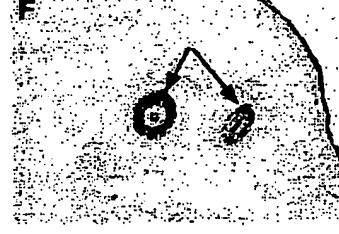


Fig. 26G

Fig. 26H

Fig. 26I

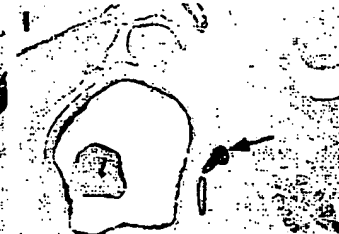
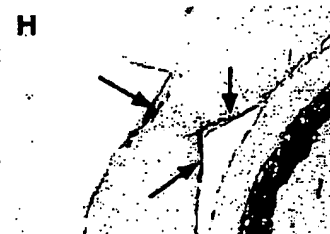


Fig. 26J

Fig. 26K

Fig. 26L



Fig. 26M

Fig. 26N

Fig. 26O



Fig. 26P

Fig. 26Q



Fig. 26R

Fig. 26S

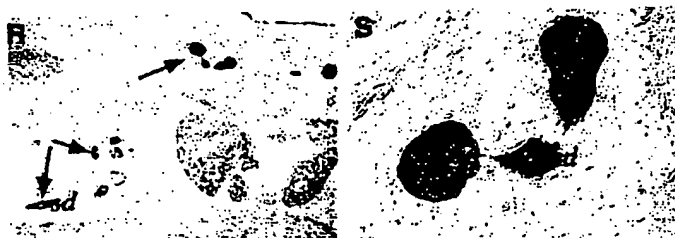


Fig. 26T

Fig. 26U

Fig. 26V

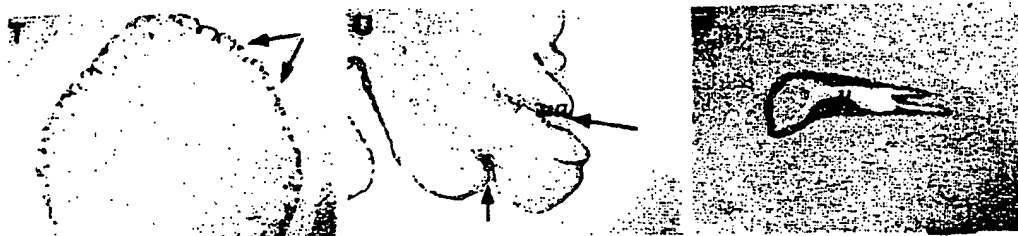


Fig. 27A



Fig. 27B

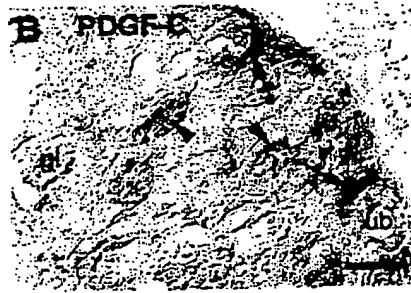


Fig. 27C



Fig. 27D



Fig. 27E



Fig. 27F



Fig. 28A



Fig. 28B

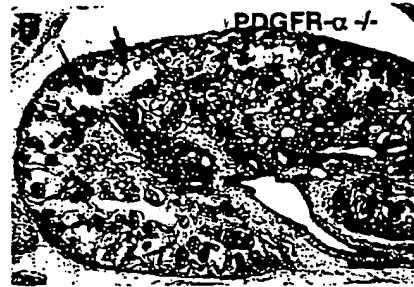


Fig. 28C

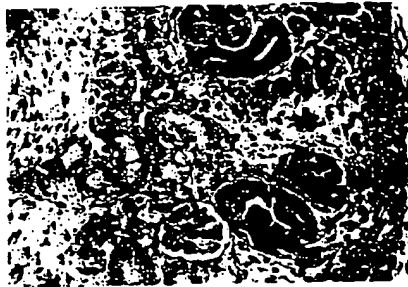


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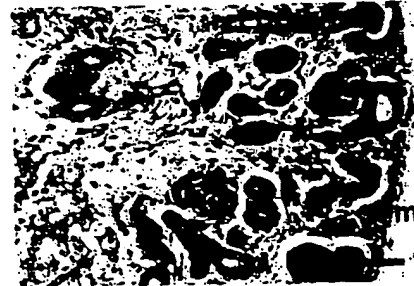


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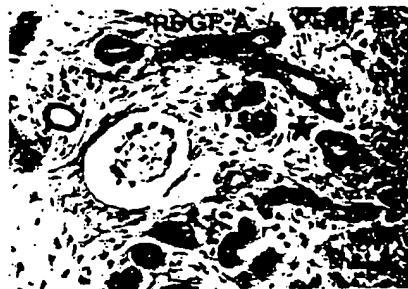
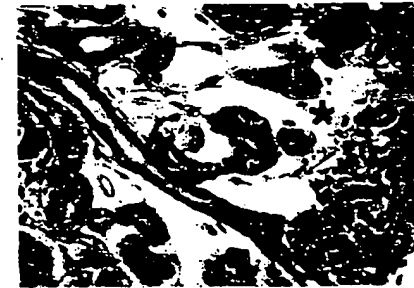


Fig. 28F



## SEQUENCE LISTING

&lt;110&gt; ERIKSSON, Ulf

AASE, Karin

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UUTELA, Marko

ALITALO, Kari

OESTMAN, Arne

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BETSHOLTZ, Christer

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Ser Pro Arg Phe Pro His Thr Tyr Pro Arg Asn Thr Val Leu Val Trp  
 35 40 45

Arg Leu Val Ala Val Glu Glu Asn Val Trp Ile Gln Leu Thr Phe Asp  
 50 55 60

Glu Arg Phe Gly Leu Glu Asp Pro Glu Asp Asp Ile Cys Lys Tyr Asp  
 65 70 75 80

Phe Val Glu Val Glu Glu Pro Ser Asp Gly Thr Ile Leu Gly Arg Trp  
 85 90 95

Cys Gly Ser Gly Thr Val Pro Gly Lys Gln Ile Ser Lys Gly Asn Gln  
 100 105 110

Ile Arg Ile Arg Phe Val Ser Asp Glu Tyr Phe Pro Ser Glu Pro Gly  
 115 120 125

Phe Cys Ile His Tyr Asn Ile Val Met Pro Gln Phe Thr Glu Ala Val  
 130 135 140

Ser Pro Ser Val Leu Pro Pro Ser Ala Leu Pro Leu Asp Leu Leu Asn  
 145 150 155 160

Asn Ala Ile Thr Ala Phe Ser Thr Leu Glu Asp Leu Ile Arg Tyr Leu  
 165 170 175

Glu Pro Glu Arg Trp Gln Leu Asp Leu Glu Asp Leu Tyr Arg Pro Thr  
 180 185 190

Trp Gln Leu Leu Gly Lys Ala Phe Val Phe Gly Arg Lys Ser Arg Val  
 195 200 205

Val Asp Leu Asn Leu Leu Thr Glu Glu Val Arg Leu Tyr Ser Cys Thr  
 210 215 220

Pro Arg Asn Phe Ser Val Ser Ile Arg Glu Glu Leu Lys Arg Thr Asp  
 225 230 235 240

Thr Ile Phe Trp Pro Gly Cys Leu Leu Val Lys Arg Cys Gly Gly Asn  
 245 250 255

Cys Ala Cys Cys Leu His Asn Cys Asn Glu Cys Gln Cys Val Pro Ser  
 260 265 270

Lys Val Thr Lys Lys Tyr His Glu Val Leu Gln Leu Arg Pro Lys Thr  
 275 280 285

Gly Val Arg Gly Leu His Lys Ser Leu Thr Asp Val Ala Leu Glu His  
 290 295 300

His Glu Glu Cys Asp Cys Val Cys Arg Gly Ser Thr Gly Gly  
 305 310 315

<210> 6

<211> 1474

<212> DNA

<213> Murinae gen. sp.

<400> 6

cacctggaga cacagaagag ggctctagga aaaattttgg atggggatta tgtggaaact 60  
 accctgcgat tctctgctgc cagagccggc caggcgcttc caccgcagcg cagcctttcc 120  
 ccgggctggg ctgagccttg gagtcgtcgc ttccccagtg cccgccgcga gtgagccctc 180  
 gccccagtcg gccaaatgct cctcctcggc ctccctcctgc tgacatctgc cctggccggc 240  
 caaagaacgg ggactcgggc tgagtccaac ctgagcagca agttgcagct ctccagcgac 300  
 aaggaacaga acggagtgca agatccccgg catgagagag ttgtcactat atctggtaat 360  
 gggagcatcc acagcccgaa gtttcctcat acgtacccaa gaaatatggt gctggtgtgg 420  
 agattagtgg cagtagatga aaatgtgcgg atccagctga catttgatga gagatttggg 480  
 ctggaagatc cagaagacga tatatgcaag tatgattttg tagaagttga ggagcccagt 540  
 gatggaagtg ttttaggacg ctggtgtggt tctgggactg tgccaggaaa gcagacttct 600  
 aaaggaaatc atatcaggat aagatttgta tctgatgagt attttccatc tgaacccgga 660  
 ttctgcatcc actacagtat tatcatgcca caagtcacag aaaccacgag tccttcggtg 720  
 ttgccccctt catctttgtc attggacctg ctcaacaatg ctgtgactgc cttcagtacc 780  
 ttggaagagc tgattcggta cctagagcca gatcgatggc aggtggactt ggacagcctc 840  
 tacaagccaa catggcagct tttgggcaag gctttcctgt atgggaaaaa aagcaaagtg 900  
 gtgaatctga atctcctcaa ggaagaggta aaactctaca gctgcacacc ccggaacttc 960  
 tcagtgtcca tacgggaaga gctaaagagg acagatacca tattctggcc aggttgtctc 1020

ctggtcaagc gctgtggagg aaattgtgcc tgtgtctcc ataattgcaa tgaatgtcag 1080  
 tgtgtccac gtaaagtac aaaaaagtac catgagggtcc ttcagttgag accaaaaact 1140  
 ggagtcagg gattgcataa gtcactcact gatgtggctc tggaacacca cgaggaatgt 1200  
 gactgtgtgt gtagaggaaa cgcaggaggg taactgcagc cttcgtagca gcacacgtga 1260  
 gactggcat tctgtgtacc cccacaagca accttcaccc ccaccagcgt tggccgcagg 1320  
 gctctcagct gctgatgctg gctatggtaa agatcttact cgtctccaac caaattctca 1380  
 gttgtttgct tcaatagcct tccccgcag gacttcaagt gtcttctaaa agaccagagg 1440  
 caccaanagg agtcaatcac aaagcactgc accg 1474

<210> 7

<211> 345

<212> PRT

<213> Murinae gen. sp.

<400> 7

Met Leu Leu Leu Gly Leu Leu Leu Leu Thr Ser Ala Leu Ala Gly Gln  
 1 5 10 15

Arg Thr Gly Thr Arg Ala Glu Ser Asn Leu Ser Ser Lys Leu Gln Leu  
 20 25 30

Ser Ser Asp Lys Glu Gln Asn Gly Val Gln Asp Pro Arg His Glu Arg  
 35 40 45

Val Val Thr Ile Ser Gly Asn Gly Ser Ile His Ser Pro Lys Phe Pro  
 50 55 60

His Thr Tyr Pro Arg Asn Met Val Leu Val Trp Arg Leu Val Ala Val  
 65 70 75 80

Asp Glu Asn Val Arg Ile Gln Leu Thr Phe Asp Glu Arg Phe Gly Leu  
 85 90 95

Glu Asp Pro Glu Asp Asp Ile Cys Lys Tyr Asp Phe Val Glu Val Glu  
 100 105 110

Glu Pro Ser Asp Gly Ser Val Leu Gly Arg Trp Cys Gly Ser Gly Thr  
 115 120 125

Val Pro Gly Lys Gln Thr Ser Lys Gly Asn His Ile Arg Ile Arg Phe  
 130 135 140

Val Ser Asp Glu Tyr Phe Pro Ser Glu Pro Gly Phe Cys Ile His Tyr  
 145 150 155 160

Ser Ile Ile Met Pro Gln Val Thr Glu Thr Thr Ser Pro Ser Val Leu  
 165 170 175

Pro Pro Ser Ser Leu Ser Leu Asp Leu Leu Asn Asn Ala Val Thr Ala  
180 185 190

Phe Ser Thr Leu Glu Glu Leu Ile Arg Tyr Leu Glu Pro Asp Arg Trp  
195 200 205

Gln Val Asp Leu Asp Ser Leu Tyr Lys Pro Thr Trp Gln Leu Leu Gly  
210 215 220

Lys Ala Phe Leu Tyr Gly Lys Lys Ser Lys Val Val Asn Leu Asn Leu  
225 230 235 240

Leu Lys Glu Glu Val Lys Leu Tyr Ser Cys Thr Pro Arg Asn Phe Ser  
245 250 255

Val Ser Ile Arg Glu Glu Leu Lys Arg Thr Asp Thr Ile Phe Trp Pro  
260 265 270

Gly Cys Leu Leu Val Lys Arg Cys Gly Gly Asn Cys Ala Cys Cys Leu  
275 280 285

His Asn Cys Asn Glu Cys Gln Cys Val Pro Arg Lys Val Thr Lys Lys  
290 295 300

Tyr His Glu Val Leu Gln Leu Arg Pro Lys Thr Gly Val Lys Gly Leu  
305 310 315 320

His Lys Ser Leu Thr Asp Val Ala Leu Glu His His Glu Glu Cys Asp  
325 330 335

Cys Val Cys Arg Gly Asn Ala Gly Gly  
340 345

<210> 8

<211> 192

<212> PRT

<213> Homo sapiens

<400> 8

Met Asn Phe Leu Leu Ser Trp Val His Trp Ser Leu Ala Leu Leu Leu  
1 5 10 15

Tyr Leu His His Ala Lys Trp Ser Gln Ala Ala Pro Met Ala Glu Gly  
20 25 30

Gly Gly Gln Asn His His Glu Val Val Lys Phe Met Asp Val Tyr Gln  
35 40 45

Arg Ser Tyr Cys His Pro Ile Glu Thr Leu Val Asp Ile Phe Gln Glu  
 50 55 60  
 Tyr Pro Asp Glu Ile Glu Tyr Ile Phe Lys Pro Ser Cys Val Pro Leu  
 65 70 75 80  
 Met Arg Cys Gly Gly Cys Cys Asn Asp Glu Gly Leu Glu Cys Val Pro  
 85 90 95  
 Thr Glu Glu Ser Asn Ile Thr Met Gln Ile Met Arg Ile Lys Pro His  
 100 105 110  
 Gln Gly Gln His Ile Gly Glu Met Ser Phe Leu Gln His Asn Lys Cys  
 115 120 125  
 Glu Cys Arg Pro Lys Lys Asp Arg Ala Arg Gln Glu Asn Pro Cys Gly  
 130 135 140  
 Pro Cys Ser Ser Glu Arg Arg Lys His Leu Phe Val Gln Asp Pro Gln  
 145 150 155 160  
 Thr Cys Lys Cys Ser Cys Lys Asn Thr Asp Ser Arg Cys Lys Ala Arg  
 165 170 175  
 Gln Leu Glu Leu Asn Glu Arg Thr Cys Arg Cys Asp Lys Pro Arg Arg  
 180 185 190

&lt;210&gt; 9

&lt;211&gt; 170

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 9

Met Pro Val Met Arg Leu Phe Pro Cys Phe Leu Gln Leu Leu Ala Gly  
 1 5 10 15  
 Leu Ala Leu Pro Ala Val Pro Pro Gln Gln Trp Ala Leu Ser Ala Gly  
 20 25 30  
 Asn Gly Ser Ser Glu Val Glu Val Val Pro Phe Gln Glu Val Trp Gly  
 35 40 45  
 Arg Ser Tyr Cys Arg Ala Leu Glu Arg Leu Val Asp Val Val Ser Glu

50                      55                      60  
 Tyr Pro Ser Glu Val Glu His Met Phe Ser Pro Ser Cys Val Ser Leu  
 65                      70                      75                      80  
 Leu Arg Cys Thr Gly Cys Cys Gly Asp Glu Asp Leu His Cys Val Pro  
                     85                      90                      95  
 Val Glu Thr Ala Asn Val Thr Met Gln Leu Leu Lys Ile Arg Ser Gly  
                     100                      105                      110  
 Asp Arg Pro Ser Tyr Val Glu Leu Thr Phe Ser Gln His Val Arg Cys  
                     115                      120                      125  
 Glu Cys Arg Pro Leu Arg Glu Lys Met Lys Pro Glu Arg Arg Arg Pro  
                     130                      135                      140  
 Lys Gly Arg Gly Lys Arg Arg Arg Glu Asn Gln Arg Pro Thr Asp Cys  
 145                      150                      155                      160  
 His Leu Cys Gly Asp Ala Val Pro Arg Arg  
                     165                      170

&lt;210&gt; 10

&lt;211&gt; 188

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 10

Met Ser Pro Leu Leu Arg Arg Leu Leu Leu Ala Ala Leu Leu Gln Leu  
                     1                      5                      10                      15  
 Ala Pro Ala Gln Ala Pro Val Ser Gln Pro Asp Ala Pro Gly His Gln  
                     20                      25                      30  
 Arg Lys Val Val Ser Trp Ile Asp Val Tyr Thr Arg Ala Thr Cys Gln  
                     35                      40                      45  
 Pro Arg Glu Val Val Val Pro Leu Thr Val Glu Leu Met Gly Thr Val  
                     50                      55                      60  
 Ala Lys Gln Leu Val Pro Ser Cys Val Thr Val Gln Arg Cys Gly Gly  
 65                      70                      75                      80  
 Cys Cys Pro Asp Asp Gly Leu Glu Cys Val Pro Thr Gly Gln His Gln  
                     85                      90                      95

Val Arg Met Gln Ile Leu Met Ile Arg Tyr Pro Ser Ser Gln Leu Gly  
100 105 110

Glu Met Ser Leu Glu Glu His Ser Gln Cys Glu Cys Arg Pro Lys Lys  
115 120 125

Lys Asp Ser Ala Val Lys Pro Asp Ser Pro Arg Pro Leu Cys Pro Arg  
130 135 140

Cys Thr Gln His His Gln Arg Pro Asp Pro Arg Thr Cys Arg Cys Arg  
145 150 155 160

Cys Arg Arg Arg Ser Phe Leu Arg Cys Gln Gly Arg Gly Leu Glu Leu  
165 170 175

Asn Pro Asp Thr Cys Arg Cys Arg Lys Leu Arg Arg  
180 185

<210> 11

<211> 133

<212> PRT

<213> Homo sapiens

<400> 11

Met Lys Leu Leu Val Gly Ile Leu Val Ala Val Cys Leu His Gln Tyr  
1 5 10 15

Leu Leu Asn Ala Asp Ser Asn Thr Lys Gly Trp Ser Glu Val Leu Lys  
20 25 30

Gly Ser Glu Cys Lys Pro Arg Pro Ile Val Val Pro Val Ser Glu Thr  
35 40 45

His Pro Glu Leu Thr Ser Gln Arg Phe Asn Pro Pro Cys Val Thr Leu  
50 55 60

Met Arg Cys Gly Gly Cys Cys Asn Asp Glu Ser Leu Glu Cys Val Pro  
65 70 75 80

Thr Glu Glu Val Asn Val Ser Met Glu Leu Leu Gly Ala Ser Gly Ser  
85 90 95

Gly Ser Asn Gly Met Gln Arg Leu Ser Phe Val Glu His Lys Lys Cys  
100 105 110

Asp Cys Arg Pro Arg Phe Thr Thr Thr Pro Pro Thr Thr Thr Arg Pro  
115 120 125

Pro Arg Arg Arg Arg  
130

<210> 12

<211> 419

<212> PRT

<213> Homo sapiens

<400> 12

Met His Leu Leu Gly Phe Phe Ser Val Ala Cys Ser Leu Leu Ala Ala  
1 5 10 15

Ala Leu Leu Pro Gly Pro Arg Glu Ala Pro Ala Ala Ala Ala Phe  
20 25 30

Glu Ser Gly Leu Asp Leu Ser Asp Ala Glu Pro Asp Ala Gly Glu Ala  
35 40 45

Thr Ala Tyr Ala Ser Lys Asp Leu Glu Glu Gln Leu Arg Ser Val Ser  
50 55 60

Ser Val Asp Glu Leu Met Thr Val Leu Tyr Pro Glu Tyr Trp Lys Met  
65 70 75 80

Tyr Lys Cys Gln Leu Arg Lys Gly Gly Trp Gln His Asn Arg Glu Gln  
85 90 95

Ala Asn Leu Asn Ser Arg Thr Glu Glu Thr Ile Lys Phe Ala Ala Ala  
100 105 110

His Tyr Asn Thr Glu Ile Leu Lys Ser Ile Asp Asn Glu Trp Arg Lys  
115 120 125

Thr Gln Cys Met Pro Arg Glu Val Cys Ile Asp Val Gly Lys Glu Phe  
130 135 140

Gly Val Ala Thr Asn Thr Phe Phe Lys Pro Pro Cys Val Ser Val Tyr  
145 150 155 160

Arg Cys Gly Gly Cys Cys Asn Ser Glu Gly Leu Gln Cys Met Asn Thr  
165 170 175

Ser Thr Ser Tyr Leu Ser Lys Thr Leu Phe Glu Ile Thr Val Pro Leu  
180 185 190

Ser Gln Gly Pro Lys Pro Val Thr Ile Ser Phe Ala Asn His Thr Ser



195	200	205
Cys Arg Cys Met Ser Lys Leu Asp Val Tyr Arg Gln Val His Ser Ile		
210	215	220
Ile Arg Arg Ser Leu Pro Ala Thr Leu Pro Gln Cys Gln Ala Ala Asn		
225	230	235 240
Lys Thr Cys Pro Thr Asn Tyr Met Trp Asn Asn His Ile Cys Arg Cys		
	245	250 255
Leu Ala Gln Glu Asp Phe Met Phe Ser Ser Asp Ala Gly Asp Asp Ser		
	260	265 270
Thr Asp Gly Phe His Asp Ile Cys Gly Pro Asn Lys Glu Leu Asp Glu		
	275	280 285
Glu Thr Cys Gln Cys Val Cys Arg Ala Gly Leu Arg Pro Ala Ser Cys		
	290	295 300
Gly Pro His Lys Glu Leu Asp Arg Asn Ser Cys Gln Cys Val Cys Lys		
305	310	315 320
Asn Lys Leu Phe Pro Ser Gln Cys Gly Ala Asn Arg Glu Phe Asp Glu		
	325	330 335
Asn Thr Cys Gln Cys Val Cys Lys Arg Thr Cys Pro Arg Asn Gln Pro		
	340	345 350
Leu Asn Pro Gly Lys Cys Ala Cys Glu Cys Thr Glu Ser Pro Gln Lys		
	355	360 365
Cys Leu Leu Lys Gly Lys Lys Phe His His Gln Thr Cys Ser Cys Tyr		
	370	375 380
Arg Arg Pro Cys Thr Asn Arg Gln Lys Ala Cys Glu Pro Gly Phe Ser		
385	390	395 400
Tyr Ser Glu Glu Val Cys Arg Cys Val Pro Ser Tyr Trp Lys Arg Pro		
	405	410 415
Gln Met Ser		

<210> 13  
 <211> 358  
 <212> PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 13

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Met Tyr Gly Glu Trp Gly Met Gly Asn Ile Leu Met Met Phe His Val
 1           5           10           15

Tyr Leu Val Gln Gly Phe Arg Ser Glu His Gly Pro Val Lys Asp Phe
          20           25           30

Ser Phe Glu Arg Ser Ser Arg Ser Met Leu Glu Arg Ser Glu Gln Gln
          35           40           45

Ile Arg Ala Ala Ser Ser Leu Glu Glu Leu Leu Gln Ile Ala His Ser
          50           55           60

Glu Asp Trp Lys Leu Trp Arg Cys Arg Leu Lys Leu Lys Ser Leu Ala
          65           70           75           80

Ser Met Asp Ser Arg Ser Ala Ser His Arg Ser Thr Arg Phe Ala Ala
          85           90           95

Thr Phe Tyr Asp Thr Glu Thr Leu Lys Val Ile Asp Glu Glu Trp Gln
          100          105          110

Arg Thr Gln Cys Ser Pro Arg Glu Thr Cys Val Glu Val Ala Ser Glu
          115          120          125

Leu Gly Lys Thr Thr Asn Thr Phe Phe Lys Pro Pro Cys Val Asn Val
          130          135          140

Phe Arg Cys Gly Gly Cys Cys Asn Glu Glu Gly Val Met Cys Met Asn
          145          150          155          160

Thr Ser Thr Ser Tyr Ile Ser Lys Gln Leu Phe Glu Ile Ser Val Pro
          165          170          175

Leu Thr Ser Val Pro Glu Leu Val Pro Val Lys Ile Ala Asn His Thr
          180          185          190

Gly Cys Lys Cys Leu Pro Thr Gly Pro Arg His Pro Tyr Ser Ile Ile
          195          200          205

Arg Arg Ser Ile Gln Thr Pro Glu Glu Asp Glu Cys Pro His Ser Lys
          210          215          220

Lys Leu Cys Pro Ile Asp Met Leu Trp Asp Asn Thr Lys Cys Lys Cys
          225          230          235          240

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Val Leu Gln Asp Glu Thr Pro Leu Pro Gly Thr Glu Asp His Ser Tyr  
 245 250 255

Leu Gln Glu Pro Thr Leu Cys Gly Pro His Met Thr Phe Asp Glu Asp  
 260 265 270

Arg Cys Glu Cys Val Cys Lys Ala Pro Cys Pro Gly Asp Leu Ile Gln  
 275 280 285

His Pro Glu Asn Cys Ser Cys Phe Glu Cys Lys Glu Ser Leu Glu Ser  
 290 295 300

Cys Cys Gln Lys His Lys Ile Phe His Pro Asp Thr Cys Ser Cys Glu  
 305 310 315 320

Asp Arg Cys Pro Phe His Thr Arg Thr Cys Ala Ser Arg Lys Pro Ala  
 325 330 335

Cys Gly Lys His Trp Arg Phe Pro Lys Glu Thr Arg Ala Gln Gly Leu  
 340 345 350

Tyr Ser Gln Glu Asn Pro  
 355

&lt;210&gt; 14

&lt;211&gt; 211

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 14

Met Arg Thr Leu Ala Cys Leu Leu Leu Leu Gly Cys Gly Tyr Leu Ala  
 1 5 10 15

His Val Leu Ala Glu Glu Ala Glu Ile Pro Arg Glu Val Ile Glu Arg  
 20 25 30

Leu Ala Arg Ser Gln Ile His Ser Ile Arg Asp Leu Gln Arg Leu Leu  
 35 40 45

Glu Ile Asp Ser Val Gly Ser Glu Asp Ser Leu Asp Thr Ser Leu Arg  
 50 55 60

Ala His Gly Val His Ala Thr Lys His Val Pro Glu Lys Arg Pro Leu  
 65 70 75 80

Pro Ile Arg Arg Lys Arg Ser Ile Glu Glu Ala Val Pro Ala Val Cys  
 85 90 95

Lys Thr Arg Thr Val Ile Tyr Glu Ile Pro Arg Ser Gln Val Asp Pro  
100 105 110

Thr Ser Ala Asn Phe Leu Ile Trp Pro Pro Cys Val Glu Val Lys Arg  
115 120 125

Cys Thr Gly Cys Cys Asn Thr Ser Ser Val Lys Cys Gln Pro Ser Arg  
130 135 140

Val His His Arg Ser Val Lys Val Ala Lys Val Glu Tyr Val Arg Lys  
145 150 155 160

Lys Pro Lys Leu Lys Glu Val Gln Val Arg Leu Glu Glu His Leu Glu  
165 170 175

Cys Ala Cys Ala Thr Thr Ser Leu Asn Pro Asp Tyr Arg Glu Glu Asp  
180 185 190

Thr Gly Arg Pro Arg Glu Ser Gly Lys Lys Arg Lys Arg Lys Arg Leu  
195 200 205

Lys Pro Thr  
210

<210> 15

<211> 241

<212> PRT

<213> Homo sapiens

<400> 15

Met Asn Arg Cys Trp Ala Leu Phe Leu Ser Leu Cys Cys Tyr Leu Arg  
1 5 10 15

Leu Val Ser Ala Glu Gly Asp Pro Ile Pro Glu Glu Leu Tyr Glu Met  
20 25 30

Leu Ser Asp His Ser Ile Arg Ser Phe Asp Asp Leu Gln Arg Leu Leu  
35 40 45

His Gly Asp Pro Gly Glu Glu Asp Gly Ala Glu Leu Asp Leu Asn Met  
50 55 60

Thr Arg Ser His Ser Gly Gly Glu Leu Glu Ser Leu Ala Arg Gly Arg  
65 70 75 80

Arg Ser Leu Gly Ser Leu Thr Ile Ala Glu Pro Ala Met Ile Ala Glu

85

90

95

Cys Lys Thr Arg Thr Glu Val Phe Glu Ile Ser Arg Arg Leu Ile Asp  
100 105 110

Arg Thr Asn Ala Asn Phe Leu Val Trp Pro Pro Cys Val Glu Val Gln  
115 120 125

Arg Cys Ser Gly Cys Cys Asn Asn Arg Asn Val Gln Cys Arg Pro Thr  
130 135 140

Gln Val Gln Leu Arg Pro Val Gln Val Arg Lys Ile Glu Ile Val Arg  
145 150 155 160

Lys Lys Pro Ile Phe Lys Lys Ala Thr Val Thr Leu Glu Asp His Leu  
165 170 175

Ala Cys Lys Cys Glu Thr Val Ala Ala Ala Arg Pro Val Thr Arg Ser  
180 185 190

Pro Gly Gly Ser Gln Glu Gln Arg Ala Lys Thr Pro Gln Thr Arg Val  
195 200 205

Thr Ile Arg Thr Val Arg Val Arg Arg Pro Pro Lys Gly Lys His Arg  
210 215 220

Lys Phe Lys His Thr His Asp Lys Thr Ala Leu Lys Glu Thr Leu Gly  
225 230 235 240

Ala

&lt;210&gt; 16

&lt;211&gt; 182

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 16

Met Pro Gln Phe Thr Asp Cys Val Cys Arg Gly Ser Thr Gly Gly Glu  
1 5 10 15

Ala Val Ser Pro Ser Val Leu Pro Pro Ser Ala Leu Pro Leu Asp Leu  
20 25 30

Leu Asn Asn Ala Ile Thr Ala Phe Ser Thr Leu Glu Asp Leu Ile Arg  
35 40 45

Tyr Leu Glu Pro Glu Arg Trp Gln Leu Asp Leu Glu Asp Leu Tyr Arg  
 50 55 60  
 Pro Thr Trp Gln Leu Leu Gly Lys Ala Phe Val Phe Gly Arg Lys Ser  
 65 70 75 80  
 Arg Val Val Asp Leu Asn Leu Leu Thr Glu Glu Val Arg Leu Tyr Ser  
 85 90 95  
 Cys Thr Pro Arg Asn Phe Ser Val Ser Ile Arg Glu Glu Leu Lys Arg  
 100 105 110  
 Thr Asp Thr Ile Phe Trp Pro Gly Cys Leu Leu Val Lys Arg Cys Gly  
 115 120 125  
 Gly Asn Cys Ala Cys Cys Leu His Asn Cys Asn Glu Cys Gln Cys Val  
 130 135 140  
 Pro Ser Lys Val Thr Lys Lys Tyr His Glu Val Leu Gln Leu Arg Pro  
 145 150 155 160  
 Lys Thr Gly Val Arg Gly Leu His Lys Ser Leu Thr Asp Val Ala Leu  
 165 170 175  
 Glu His His Glu Glu Cys  
 180

<210> 17  
 <211> 182  
 <212> PRT  
 <213> Murinae gen. sp.

<400> 17  
 Met Pro Gln Val Thr Glu Thr Thr Ser Pro Ser Val Leu Pro Pro Ser  
 1 5 10 15  
 Ser Leu Ser Leu Asp Leu Leu Asn Asn Ala Val Thr Ala Phe Ser Thr  
 20 25 30  
 Leu Glu Glu Leu Ile Arg Tyr Leu Glu Pro Asp Arg Trp Gln Val Asp  
 35 40 45  
 Leu Asp Ser Leu Tyr Lys Pro Thr Trp Gln Leu Asp Cys Val Cys Arg  
 50 55 60  
 Gly Asn Ala Gly Gly Leu Gly Lys Ala Phe Leu Tyr Gly Lys Lys Ser  
 65 70 75 80

Lys Val Val Asn Leu Asn Leu Leu Lys Glu Glu Val Lys Leu Tyr Ser  
85 90 95

Cys Thr Pro Arg Asn Phe Ser Val Ser Ile Arg Glu Glu Leu Lys Arg  
100 105 110

Thr Asp Thr Ile Phe Trp Pro Gly Cys Leu Leu Val Lys Arg Cys Gly  
115 120 125

Gly Asn Cys Ala Cys Cys Leu His Asn Cys Asn Glu Cys Gln Cys Val  
130 135 140

Pro Arg Lys Val Thr Lys Lys Tyr His Glu Val Leu Gln Leu Arg Pro  
145 150 155 160

Lys Thr Gly Val Lys Gly Leu His Lys Ser Leu Thr Asp Val Ala Leu  
165 170 175

Glu His His Glu Glu Cys  
180

<210> 18

<211> 117

<212> PRT

<213> Murinae gen. sp.

<400> 18

Glu Arg Val Val Thr Ile Ser Gly Asn Gly Ser Ile His Ser Pro Lys  
1 5 10 15

Phe Pro His Thr Tyr Pro Arg Asn Met Val Leu Val Trp Arg Leu Val  
20 25 30

Ala Val Asp Glu Asn Val Arg Ile Gln Leu Thr Phe Asp Glu Arg Phe  
35 40 45

Gly Leu Glu Asp Pro Glu Asp Asp Ile Cys Lys Tyr Asp Phe Val Glu  
50 55 60

Val Glu Glu Pro Ser Asp Gly Ser Val Leu Gly Arg Trp Cys Gly Ser  
65 70 75 80

Gly Thr Val Pro Gly Lys Gln Thr Ser Lys Gly Asn Met Ile Arg Ile  
85 90 95

Arg Phe Val Ser Asp Glu Tyr Phe Pro Ser Glu Pro Gly Phe Cys Ile

100

105

110

His Tyr Ser Ile Ile  
115

&lt;210&gt; 19

&lt;211&gt; 117

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 19

Glu Arg Ile Ile Thr Val Ser Thr Asn Gly Ser Ile His Ser Pro Arg  
1 5 10 15

Phe Pro His Thr Tyr Pro Arg Asn Thr Val Leu Val Trp Arg Leu Val  
20 25 30

Ala Val Glu Glu Asn Val Trp Ile Gln Leu Thr Phe Asp Glu Arg Phe  
35 40 45

Gly Leu Glu Asp Pro Glu Asp Asp Ile Cys Lys Tyr Asp Phe Val Glu  
50 55 60

Val Glu Glu Pro Ser Asp Gly Thr Ile Leu Gly Arg Trp Cys Gly Ser  
65 70 75 80

Gly Thr Val Pro Gly Lys Gln Ile Ser Lys Gly Asn Gln Ile Arg Ile  
85 90 95

Arg Phe Val Ser Asp Glu Tyr Phe Pro Ser Glu Pro Gly Phe Cys Ile  
100 105 110

His Tyr Asn Ile Val  
115

&lt;210&gt; 20

&lt;211&gt; 113

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 20

Cys Gly Glu Thr Leu Gln Asp Ser Thr Gly Asn Phe Ser Ser Pro Glu  
1 5 10 15

Tyr Pro Asn Gly Tyr Ser Ala His Met His Cys Val Trp Arg Ile Ser  
20 25 30



Val Thr Pro Gly Glu Lys Ile Ile Leu Asn Phe Thr Ser Leu Asp Leu  
 35 40 45

Tyr Arg Ser Arg Leu Cys Trp Tyr Asp Tyr Val Glu Val Arg Asp Gly  
 50 55 60

Phe Trp Arg Lys Ala Pro Leu Arg Gly Arg Phe Cys Gly Ser Lys Leu  
 65 70 75 80

Pro Glu Pro Ile Val Ser Thr Asp Ser Arg Leu Trp Val Glu Phe Arg  
 85 90 95

Ser Ser Ser Asn Trp Val Gly Lys Gly Phe Phe Ala Val Tyr Glu Ala  
 100 105 110

Ile

<210> 21  
 <211> 112  
 <212> PRT  
 <213> Homo sapiens

<400> 21  
 Cys Gly Gly Asp Val Lys Lys Asp Tyr Gly His Ile Gln Ser Pro Asn  
 1 5 10 15

Tyr Pro Asp Asp Tyr Arg Pro Ser Lys Val Cys Ile Trp Arg Ile Gln  
 20 25 30

Val Ser Glu Gly Phe His Val Gly Leu Thr Phe Gln Ser Phe Glu Ile  
 35 40 45

Glu Arg Met Asp Ser Cys Ala Tyr Asp Tyr Leu Glu Val Arg Asp Gly  
 50 55 60

His Ser Glu Ser Ser Thr Leu Ile Gly Arg Tyr Cys Gly Tyr Glu Lys  
 65 70 75 80

Pro Asp Asp Ile Lys Ser Thr Ser Ser Arg Leu Trp Leu Lys Phe Val  
 85 90 95

Ser Asp Gly Ser Ile Asn Lys Ala Gly Phe Ala Val Asn Phe Phe Lys  
 100 105 110

<210> 22  
 <211> 113  
 <212> PRT  
 <213> Homo sapiens

<400> 22  
 Cys Gly Gly Phe Leu Thr Lys Leu Asn Gly Ser Ile Thr Ser Pro Gly  
           1                  5                  10                  15  
 Trp Pro Lys Glu Tyr Pro Pro Asn Lys Asn Cys Ile Trp Gln Leu Val  
                   20                  25                  30  
 Ala Pro Thr Gln Tyr Arg Ile Ser Leu Gln Phe Asp Phe Phe Glu Thr  
                   35                  40                  45  
 Glu Gly Asn Asp Val Cys Lys Tyr Asp Phe Val Glu Val Arg Ser Gly  
           50                  55                  60  
 Leu Thr Ala Asp Ser Lys Leu His Gly Lys Phe Cys Gly Ser Glu Lys  
           65                  70                  75                  80  
 Pro Glu Val Ile Thr Ser Gln Tyr Asn Asn Met Arg Val Glu Pro Lys  
                   85                  90                  95  
 Ser Asp Asn Thr Val Ser Lys Lys Gly Phe Lys Ala His Phe Phe Ser  
           100                  105                  110  
 Glu

<210> 23  
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 <212> PRT  
 <213> Homo sapiens

<400> 23  
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 Tyr Pro His Ser Tyr His Pro Ser Glu Lys Cys Glu Trp Leu Ile Gln  
                   20                  25                  30  
 Ala Pro Asp Pro Tyr Gln Arg Ile Met Ile Asn Phe Asn Pro His Phe  
           35                  40                  45

Asp Leu Glu Asp Arg Asp Cys Lys Tyr Asp Tyr Val Glu Val Phe Asp  
50 55 60

Gly Glu Asn Glu Asn Gly His Phe Arg Gly Lys Phe Cys Gly Lys Ile  
65 70 75 80

Ala Pro Pro Pro Val Val Ser Ser Gly Pro Phe Leu Phe Ile Lys Phe  
85 90 95

Val Ser Asp Tyr Glu Thr His Gly Ala Gly Phe Ser Ile Arg Tyr Glu  
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Ile

<210> 24

<211> 119

<212> PRT

<213> Homo sapiens

<400> 24

Cys Ser Gln Asn Tyr Thr Thr Pro Ser Gly Val Ile Lys Ser Pro Gly  
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Phe Pro Glu Lys Tyr Pro Asn Ser Leu Glu Cys Thr Tyr Ile Val Phe  
20 25 30

Ala Pro Lys Met Ser Glu Ile Ile Leu Glu Phe Glu Ser Phe Asp Leu  
35 40 45

Glu Pro Asp Ser Asn Pro Pro Gly Gly Met Phe Cys Arg Tyr Asp Arg  
50 55 60

Leu Glu Ile Trp Asp Gly Phe Pro Asp Val Gly Pro His Ile Gly Arg  
65 70 75 80

Tyr Cys Gly Gln Lys Thr Pro Gly Arg Ile Arg Ser Ser Ser Gly Ile  
85 90 95

Leu Ser Met Val Phe Tyr Thr Asp Ser Ala Ile Ala Lys Glu Gly Phe  
100 105 110

Ser Ala Asn Tyr Ser Val Leu  
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19

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20

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<400> 28  
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19

<210> 29  
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<213> Murinae gen. sp.

<400> 30  
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Pro Gln His Glu Arg Cys  
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<212> PRT  
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1 5 10 15

Arg Leu Tyr Ser Cys  
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<210> 33  
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<400> 33  
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<210> 34  
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<212> DNA  
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29

&lt;210&gt; 36

&lt;211&gt; 61

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;400&gt; 36

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&lt;210&gt; 37

&lt;211&gt; 21

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 37

Gly Arg Lys Ser Arg Val Val Asp Leu Asn Leu Leu Thr Glu Glu Val  
1 5 10 15

Arg Leu Tyr Ser Cys

20

&lt;210&gt; 38

&lt;211&gt; 26

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;220&gt;

<223> Forward PCR primer from the human PDGF-C 430 bp  
cDNA fragment encoding the CUB domain which  
includes a BamHI site

&lt;400&gt; 38

cgggatcccg aatccaacct gagtag

26

&lt;210&gt; 39

&lt;211&gt; 60

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;220&gt;

<223> Reverse PCR primer from the human PDGF-C 430 bp  
cDNA fragment encoding the CUB domain which  
includes a EcoRI site and sequences coding for a  
C-terminal 6X His tag preceded by an enterokinase

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PCT/US99/22668

site

<400> 39

ccggaattcc taatggtgat ggtgatgatg ttgtcatcg tcgtcgacaa tgtttagtg 60

# PATENT COOPERATION TREATY

## PCT

### DECLARATION OF NON-ESTABLISHMENT OF INTERNATIONAL SEARCH REPORT

(PCT Article 17(2)(a) and Rule 39)

Applicant's or agent's file reference 1064/44740PC	<b>IMPORTANT DECLARATION</b>	Date of mailing (day/month/year)
International application No. PCT/US99/22668	International filing date (day/month/year) 30 SEPTEMBER 1999	(Earliest) Priority Date (day/month/year) 30 SEPTEMBER 1998
International Patent Classifying (IPC) or both national classification and IPC		
Applicant LUDWIG INSTITUTE FOR CANCER RESEARCH		

This International Searching Authority hereby declares, according to Article 17(2)(a), that no international search report will be established on the international application for the reasons indicated below.

1. ☐ The subject matter of the international application relates to:
  - a. ☐ scientific theories.
  - b. ☐ mathematical theories.
  - c. ☐ plant varieties.
  - d. ☐ animal varieties.
  - e. ☐ essentially biological processes for the production of plants and animals, other than microbiological processes and the products of such processes.
  - f. ☐ schemes, rules or methods of doing business.
  - g. ☐ schemes, rules or methods of performing purely mental acts.
  - h. ☐ schemes, rules or methods of playing games.
  - i. ☐ methods for treatment of the human body by surgery or therapy.
  - j. ☐ methods for treatment of the animal body by surgery or therapy.
  - k. ☐ diagnostic methods practiced on the human or animal body.
  - l. ☐ mere presentations of information.
  - m. ☐ computer programs for which this International Searching Authority is not equipped to search prior art.
2. ☐ The failure of the following parts of the international application to comply with prescribed requirements prevents a meaningful search from being carried out:
 

☐ the description
 ☐ the claims
 ☐ the drawings
3. ☒ The failure of the nucleotide and/or amino acid sequence listing to comply with the prescribed requirements prevents a meaningful search from being carried out:
 

☐ it does not comply with the prescribed standard  
☒ it is not in the prescribed machine readable form
4. Further comments:  
 The CRF (computer readable form) for this case is defective. Claims which recite SEQ ID No:s or depend therefrom which cannot be searched other than by a sequence search are held unsearchable

Name and mailing address of the ISA/US  
 Commissioner of Patents and Trademarks  
 Box PCT  
 Washington, D.C. 20231

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Authorized officer

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